

**EVALUATION OF ANTI-OXIDANT AND HEPATOPROTECTIVE ACTIVITY OF
IXOREA COCCINEA LEAF EXTRACTS BY USING INVITRO AND INVIVO
MODELS**

A Dissertation submitted to
THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY,
CHENNAI– 600 032

In partial fulfillment of the requirements for the award of the Degree of
MASTER OF PHARMACY
IN
BRANCH –VI – PHARMACOLOGY

Submitted by
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KARPAGAM COLLEGE OF PHARMACY
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MAY – 2017

CERTIFICATES

CERTIFICATE

This is to certify that this dissertation entitled by **EVALUATION OF ANTI-OXIDANT AND HEPATOPROTECTIVE ACTIVITY OF *IXOREA COCCINEA* LEAF EXTRACTS BY USING *INVITRO* AND *INVIVO* MODELS** submitted by **Mr MUHAMMED SHANAVAS V K** to The Tamil Nadu Dr.M.G.R Medical University Chennai in partial fulfillment for the degree of **MASTER OF PHARMACY IN PHARMACOLOGY** is a bonafied work carried out by candidate under the guidance and supervision of **Dr.C.Senthil Kumar** Associated professor in the Department of Pharmacology , Karpagam college of Pharmacy Coimbatore-32

I have fully satisfied with his performance and work. I have forwarded this dissertation work for evaluation.

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DECLARATION

I hereby declare that this dissertation **EVALUATION OF ANTI-OXIDANT AND HEPATOPROTECTIVE ACTIVITY OF *IXOREA COCCINEA* LEAF EXTRACTS BY USING *INVITRO* AND *INVIVO* MODELS** submitted by me , in partial fulfillment of requirements for the degree of MASTER OF PHARMACY IN PHARMACOLOGY to The Tamil Nadu Dr.M.G.R Medical University , Chennai is the result of my original and independent research work carried out under the guidance of **Dr.C.Senthil Kumar.,M.Pharm** Associated Professor , Department of Pharmacology ,Karpagam College of Pharmacy , Coimbatore -32

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Pharmacology , Karpagam College of Pharmacy , Coimbatore and
evaluated by us.

Examination centre :

Date :

Internal Examiner

Convenor of Examination

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DEDICATED TO MY BELOVED PARENTS

SIBLINGS ,TEACHERS ,FRIENDS AND

ALMIGHTY

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CHAPTER I

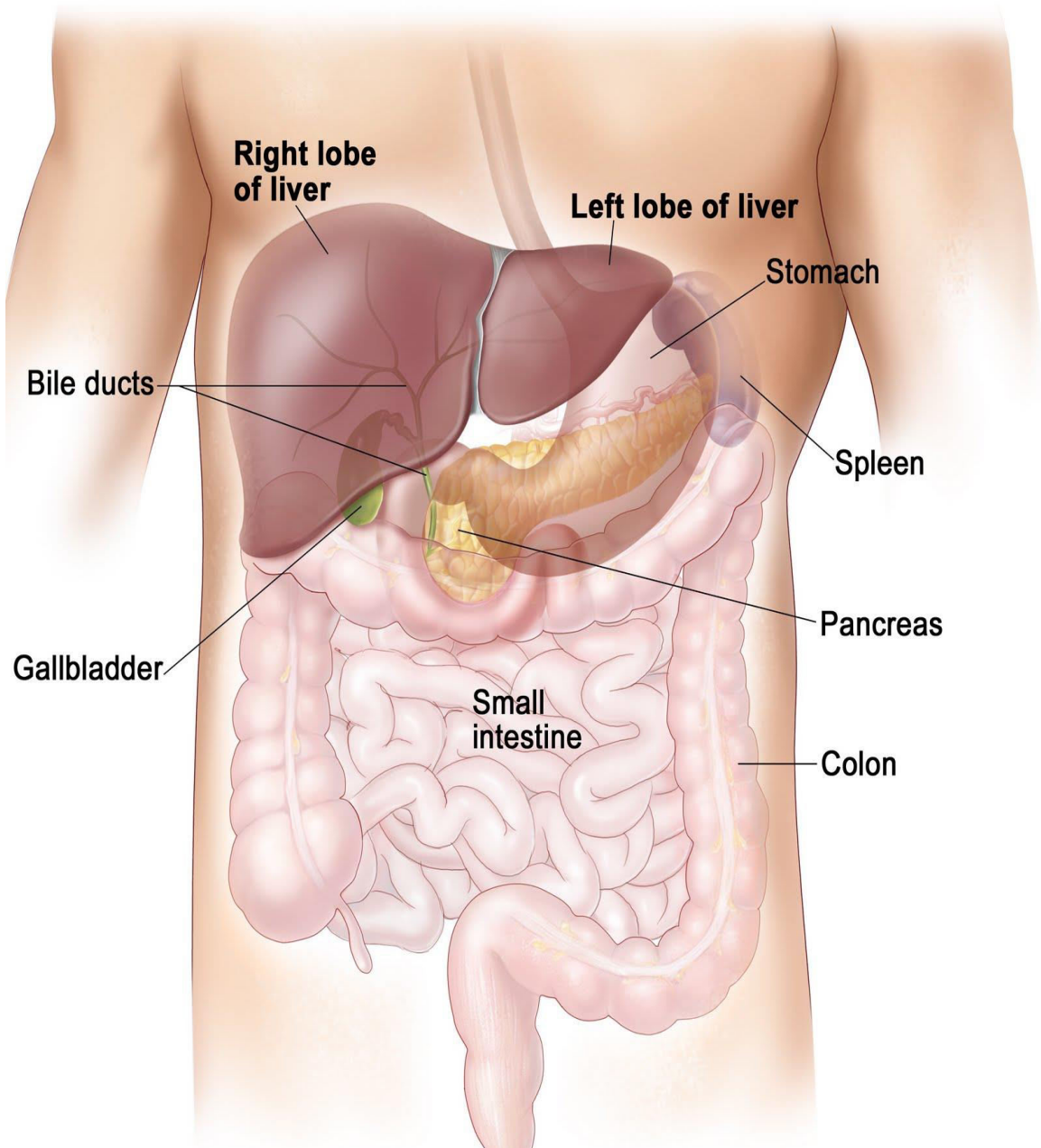
1. INTRODUCTION

1.1. Anatomy of liver

The liver is one of largest gland in the body and after the dermis¹. The liver weights about three and a half pounds (1.6 kg). It constitutes about 2.5% of adult's body weight². It is located in the upper part of the abdomen that aids in digestion and removes waste products and worn-out cells from the blood. Liver is connected to two large blood vessels which include hepatic artery and portal vein². Thirty percentage blood was pumped by the heart for one minute for body's chemical factorial organ called liver. Liver cleanses blood and processes nutritional molecule that are distributed to the tissues. Liver accept nutritional red blood by portal circulation from lungs which has filled with essential oxygen supplied to heart. It is situated in the upper part of the abdominal cavity, inferior to the diaphragm occupying the greater part of the right hypochondriac region, part of the epigastric region and extending into the left hypochondriac region. Its upper and anterior surfaces are smooth and curved to fit the under surface of the diaphragm and its posterior surface is irregular in outline³. The different types of cells propagate from the liver lobes are parenchymal and non-parenchymal type of cells. Majority (about 80%) of the liver mass is filled by parenchymal type of cells commonly known as hepatocytes. the other type non-parenchymal type cells having forty percentage of the total counts of the liver cells but it have 6.5% of its total volume². It also release about two and one-half ml of the bile in its own ducts which is delivered by a gallbladder via congested tube called the cystic duct for storage of these bile. Liver is regulated for this gland that control as to whether these incoming substances was useful for body or whether they are needless. Liver is an extremely important organ and exhibits multiple functions. Liver detoxifies for blood cells by proper fixation of bile solution via chemical modification to form less toxic substances, example alteration of ammonia to urea. Many chemical substances are inactivated by liver through modification of chemical structure. Liver convert glucose to glycogen as a

storage form of energy and it produces glucose from disaccharides and polysaccharides such as sugars, starches and protein molecules

Figure. No: 1: Location of liver in Human Digestive System⁴



The liver is situated below the diaphragm which occupy right side of hypochondriac and region in the abdominopelvic cavity. The liver is completely covered by a dense irregular connective tissue layer that lies deep to the peritoneum. It is divided into two principal lobes-a large right lobe and a smaller, wedge-shaped, left lobe separated by the falciform ligament. Right lobe is described by many anatomists to have an inferior quadrate lobes and a posterior caudate type lobes. The falciform ligament extends from undersurface of the diaphragm from the upper surface of the liver part, between two significant lobes of the liver, helped to suspend the liver. Liver is composed of several components:

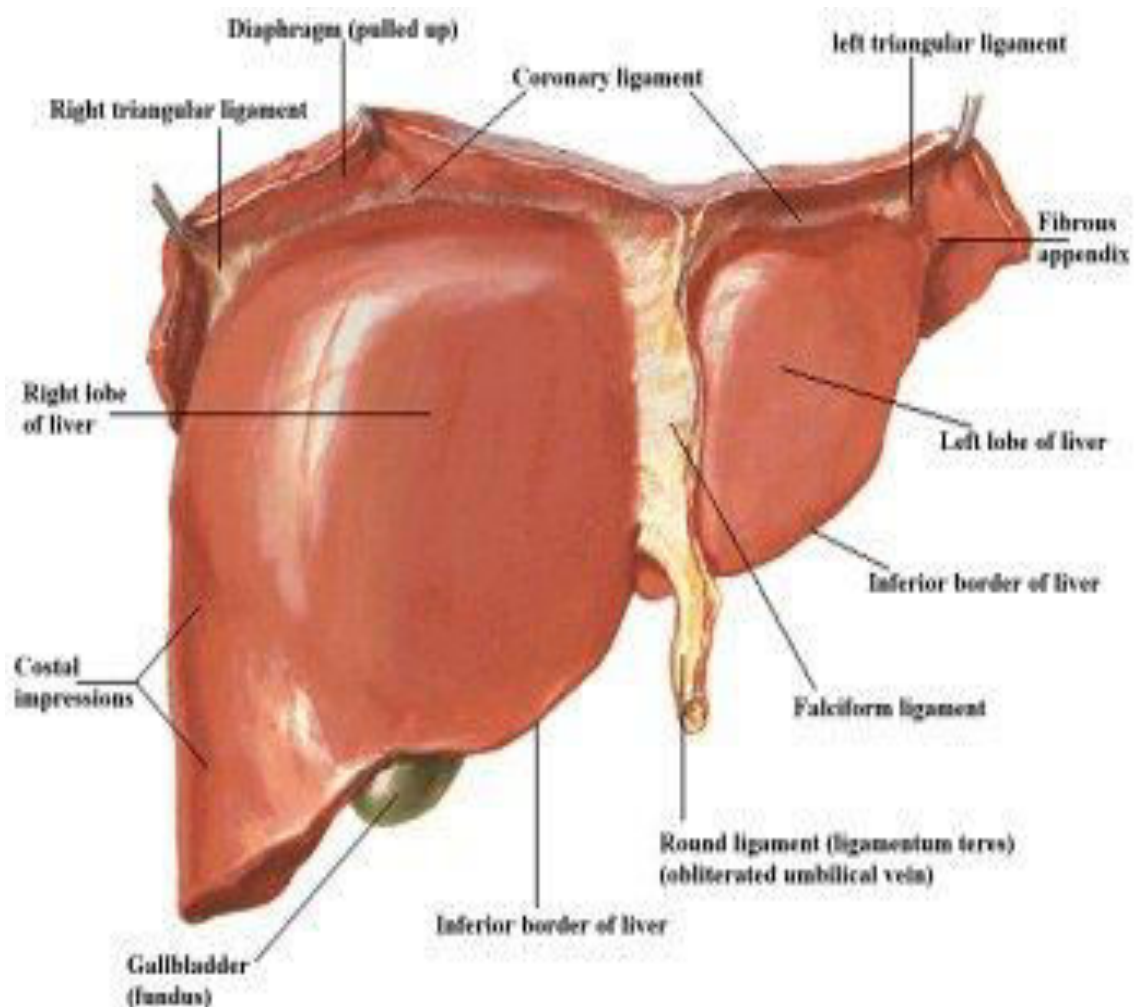
Hepatocytes are the functional cells of the liver which are arranged in pairs of columns radiating from a central vein. A wide range of metabolic, secretory and endocrine functions are performed by hepatocytes. These are specialized epithelial cells with 5-12 sides that make-up about 80% of the volume of the liver. Hepatocytes form complex three dimensional arrangements called hepatic laminae and they are the sheet of hepatocytes one cell thick lined to either side by the endothelial-lining spaces called hepatic sinusoids. Grooves inside the cell surface between neighboring hepatic cells which provide gaps for the canaliculi of hepatocytes that secrete bile. Bile is a yellow, brown, or olive-green colour type liquid which secreted from hepatic cells, which provide an excretory product and a digestive enzyme secretor.

Bile canaliculi are narrow intercellular canals that collect bile secreted by hepatocytes. From bile canaliculi, bile passes into small bile ducts. These small ducts combined by form the higher right and left hepatic duct that commonly connect and exit the liver via common hepatic duct. This common hepatic duct joins the cystic duct from the gallbladder to form the common bile duct. Bile enters the cystic duct and temporarily stored in the gallbladder. After a meal, various stimuli cause contraction of the gallbladder, which releases stored bile into the common bile duct.

Hepatic sinusoids are freely permeable capillaries about sheets of liver cells that get oxygenated blood via different branches of hepatic artery and higher amount of nutrient rich de-oxygenated blood from the branches of the hepatic

portal vein system. The hepatic portal vein helped to take the venous blood via gastrointestinal organs and spleen into the liver. Hepatic sinusoids convert and it delivered blood from the central vein blood cells. Blood flows from central veins into hepatic veins that drain into the inferior venacava. In opposite of the blood which flows toward important vein, bile flows towards the opposite side. The sinusoids are partly lined with stellate reticuloendothelial (Kupffer's) cells that destroy worn-out WBC and RBC, bacteria and other foreign substance in blood. Bile duct have part in the hepatic artery, and branch of hepatic vein are referred to as portal triad.

Figure.No:2:Liver showing right and left lobes separated by Falciform Ligament⁵



The hepatocytes, bile duct contents and hepatic sinusoids are organized into an anatomical and functional units by three different types:

Hepatic lobule: For many years, anatomy fellow are suggest that the hepatic lobule are liver's functional units. They describes the each hepatic lobule are shaped as that of a hexagonal. At its center is central vein emerging out by they form a rows of hepatocytes and hepatic sinusoidal cells. This model is suggested by detailed autopsy of the liver of the adult pigs. From the study of human liver have not predicted the hepatic lobules anatomy having a thick layers of connective tissues type.

Portal lobule: This model emphasized the endocrine function of the liver, i.e., bile release. According to the bile ducts in a portal triad is consider as the middle of the portal lobules. These portal lobule's are triangular shaped and is having a 3 imaginary straight lines which connect three central veins which are near by the portal triad. These model which are not gained wide spread acceptance.

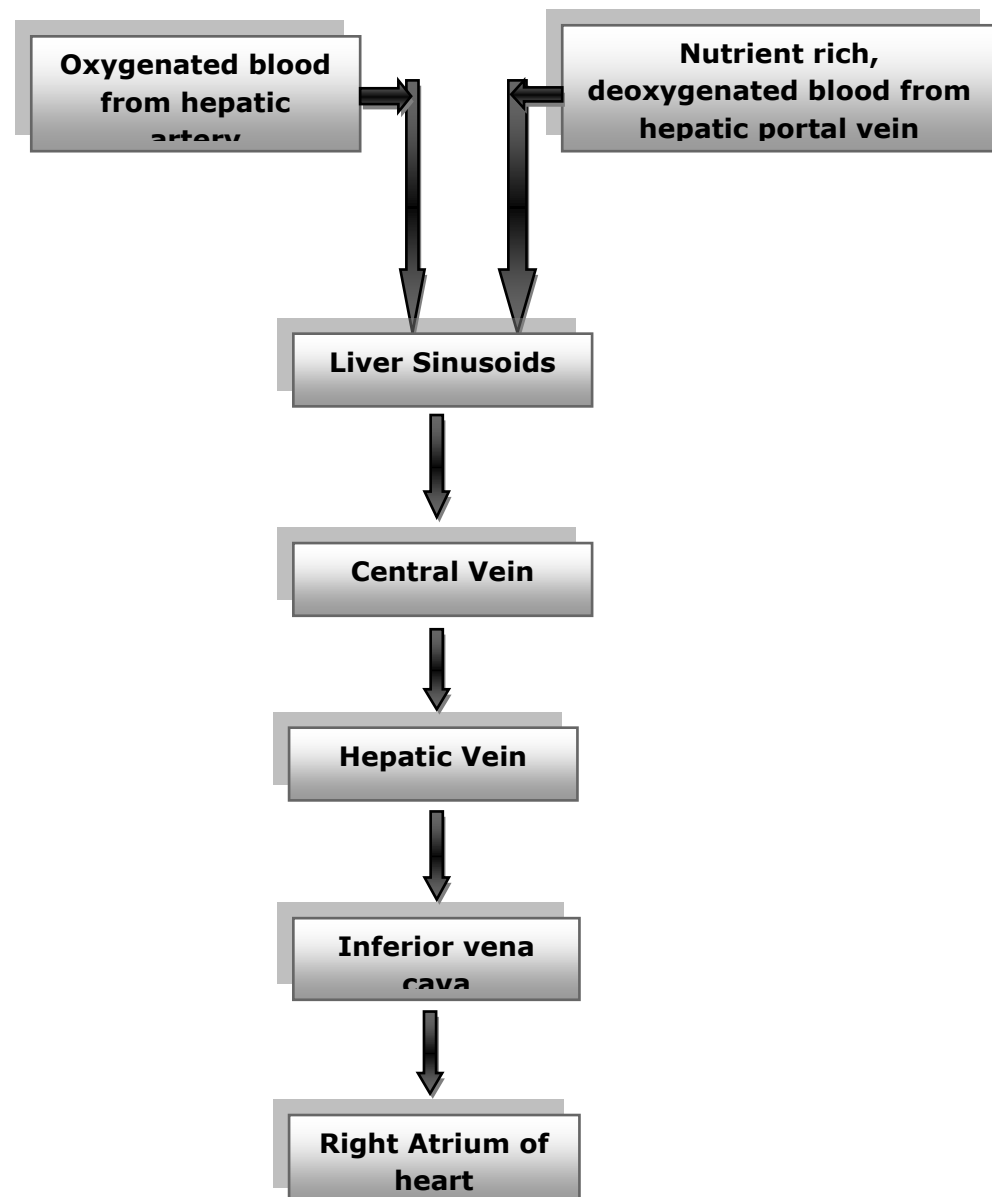
Hepatic acinus: In past years, the accepted structural and functional unit of the liver known as the hepatic acinus. These are approximately oval shape mass which includes portions of two neighboring hepatic lobules. Small axis of hepatic acinus is described by the branches of the portal triad- branches contain a hepatic artery, hepatic vein and bile ducts. Long axis of the acinus inter connected closest to short axis. Hepatocytes in the hepatic acinus are arranged in three zones from its short axis, with no sharp among them. Cells inside the zone 1 are closer to branches of portal triad and these cells are first to accept oxygen, essential food material and toxins from the receiving blood cells. These cells are gain-up glucose and save it by converting glycogen after food ingestion, so during fasting period get the energy via glycogen to glucose. These are first shows the characteristic morphological changes. Zone 1 cells are last ones to die when circulation is impaired and the first ones to regenerate. Cells in zone three are much farther from branches of portal triad and are the last to show the effects of bile blockage or presence to

toxins, the first ones effect is the abnormal circulation, retarded o rate of regeneration and evidence of fat accumulation. The cells in zone 2 show structural and functional characteristics intermediate between cells in zone 1 to 3.

1.1.1.Blood supply of liver

The liver receives blood from two sources, hepatic artery and hepatic portal vein. From hepatic artery it obtains oxygenated blood and from hepatic portal vein it receives deoxygenated blood that contains newly absorbs essential nutrients, therapeutic molecules, and possibly nonpathogenic microorganism and may receive toxins from the gastrointestinal tract. Branches of both hepatic artery and portal vein carry blood into liver sinusoids where hepatocytes extracts oxygen most of the nutrients and certain poisons. Nutrients needed by other cells and products manufactured by the hepatocytes are secreted back into the blood After blood passed inside central vein and eventually passes via hepatic vein. Branches of hepatic portal vein, artery and bile duct typically accompany each other in their distribution through liver. Collectively these three structures are called portal triad¹.

Figure. No: 3: - Hepatic blood flow path: source of blood, passing through the liver and return back to the heart³



1.1.2.Functions of liver

The liver has well over 500 functions and is known as the laboratory of human body. The liver is tied to almost all the bodily processes as it is responsible for filtration of all incoming foods and fluids⁶.

1.1.2.1. Carbohydrate metabolism

Liver is important in maintaining normal blood glucose level. Liver can break down glycogen to glucose (glycogenolysis) and release glucose into the bloodstream, when blood glucose level decreases. Liver helped the conversion of certain amino acid by glucose through lactic acid (gluconeogenesis) and convert other sugar molecules such as fructose and galactose reduced to glucose. Liver converts glucose to glycogen (glycogenesis) and also undergoes the covention of triglycerides (lipogenesis).

1.1.2.2. Lipid metabolism

Liver stores some triglycerides from fatty acids through acetyl coenzyme A known as beta oxidation. It possibly converts excess acetyl coenzyme A to ketone bodies (ketogenesis). It synthesize lipoproteins, that transportation of fatty acids, triglycerides (TG) and cholesterol from the body cells. Cholesterol is synthesized by hepatocytes and cholesterol involves the formation of bile salts.

1.1.2.3. Protein metabolism

Most of the plasma proteins, such as α and β globulins, glycol proteins (albumin and fibrinogen) are synthesized from liver cells. Also, liver enzymes can perform transamination. Liver deaminates amino acids so that they are used in the ATP synthesis or conversion from carbohydrates or fats. It converts resulting toxic ammonia into much less toxic urea for excretion in urine.

1.1.2.4. Removal of drugs and hormones

Liver can detoxify substances such as alcohol or excrete drugs like penicillin, erythromycin, and so on into bile. It is also trigger or chemically alter thyroid hormones and steroid hormones (estrogens and aldosterone).

1.1.2.5. Excretion of bilirubin

Bilirubin released from heme of red blood cells is absorbed in the liver through the blood and release to bile. Most of bilirubin in bile is metabolized in the intestine by bacteria and eliminated in feces.

1.1.2.6. Synthesis of bile salts

These are helped in small intestine functioning as an emulsification process and absorption of lipid molecules, cholesterol, phospholipids, and lipoproteins.

1.1.2.7. Storage

In addition to glycogen, liver stores water soluble and fat-soluble vitamins (A, B₁₂, D, E, and K) and essential minerals (iron and copper). Hepatocytes contain a protein called apoferritin that combines with iron to form ferritin, the form in which iron is stored in liver. The iron is secreted from the liver is essential requirement of the body.

1.1.2.8. Phagocytosis

The stellate reticuloendothelial (Kupffer's) cells of the liver phagocytize worn-out red and white blood cells and some type of the bacteria.

1.1.2.9. Activation of vitamin D

The cutaneous layer of skin, liver and kidneys essential for activation vitaminD¹.

1.1.2.10. Secretion and excretion of Bile

Bile is an incomplete synthetic substance and partially act as digestive secretion. Each day hepatic cells secrete 800-1000ml of bile. It has a PH of 7.6-8.6. Bile mainly consist water, bile salts, cholesterol and phospholipid known as lecithin, bile pigments and several ions. Principle bile pigment is bilirubin⁶.

1.1.2.11. Synthesis of vitamin A from carotene

Carotene is the pro-vitamin found in some plants for example carrots and green leaves of vegetables.

1.1.2.12. Production of heat

Liver able to create amount of energy which has a high metabolic rate and produces considerable amount of heat. It is an important heat producing organ of body³.

1.1.3.Pathology of liver⁷

All forms of injury to the liver such as microbiologic, toxic, circulatory or traumatic result in necrosis in liver. The extent of involvement of hepatic lobules necrosis varies. Accordingly, liver cell necrosis are divided into 3 types: diffuse (submissive to massive), zonal and focal.

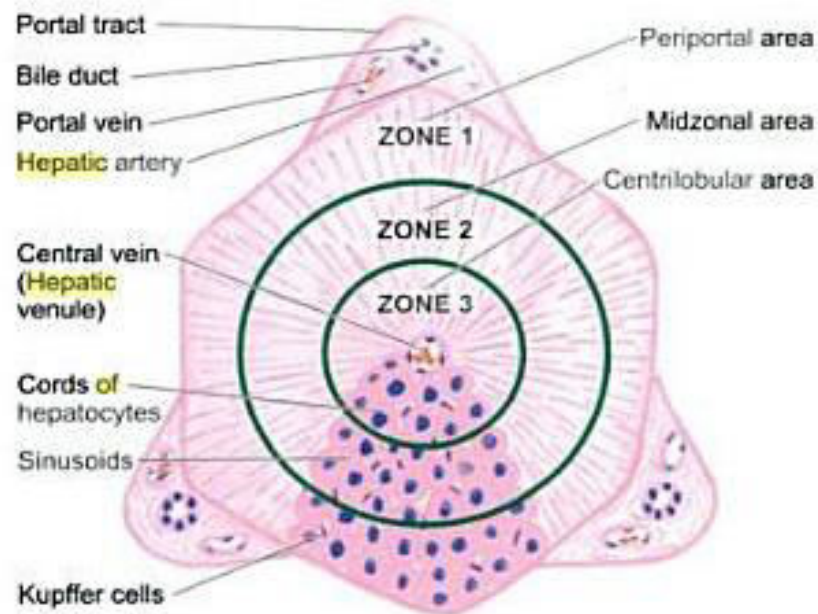
1.1.3.1.Diffuse (Submassive to massive).

When there is extensive and diffuse necrosis of the liver involving all the cells in groups of lobules, it is termed diffuse, or submissive to massive necrosis. It is most commonly caused by viral hepatitis or drug toxicity.

1.1.3. 2. Zonal necrosis.

Zonal necrosis is necrosis of hepatocytes in three different zones of the hepatic lobule as shown in the figure below.

Figure. No: 4: Histology of Hepatic lobule⁷



Accordingly, it is of three types, each type affecting respective zone is caused by different etiologic factors:

- i. **Centrilobular necrosis** is the commonest type involving hepatocytes in zone 3 (i.e. located around the central vein). Centrilobular necrosis is characteristic feature of ischemic injury such as in shock and CHF since zone 3 is farthest from the blood supply. Besides, it also occurs in poisoning with chloroform, carbon tetrachloride and certain drugs.
- ii. **Mid-zonal necrosis** is uncommon and involves zone 2 of the hepatic lobule. This pattern of necrosis is seen in yellow fever and viral hepatitis. In viral hepatitis, some of the necrosed hepatocytes of the mid-zone are transformed into acidophilic, rounded Councilman Bodies.
- iii. **Periportal (peripheral) necrosis** is seen in zone 1 involving the parenchyma closest to the arterial and portal blood supply. Since zone 1 is most well perfused, it is most vulnerable to the effects of circulating hepatotoxins e.g. in phosphorus poisoning

1.1.3.3. Focal Necrosis

This form of necrosis involves small groups of hepatocytes irregularly distributed in the hepatic lobule. Focal necrosis is most often caused by microbiological infections. These include viral hepatitis, military tuberculosis, typhoid fever and various other forms of bacteria, viral and fungal infections. Focal necrosis may also occur in drug-induced hepatitis.

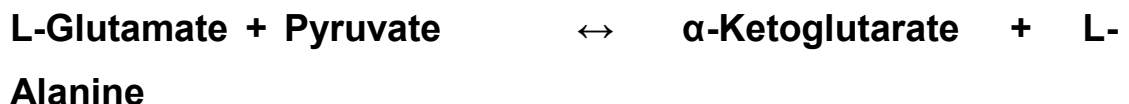
1.1.4. Enzymes involved with liver

1.1.4.1. Alanine transaminase

Alanine transaminase or ALT is a transaminase, serum glutamic–pyruvic transaminase (SGPT or also known as alanine aminotransferase (ALAT)) commonly observed in many tissues and body fluids principally in liver. ALT is released into serum as a result of tissue injury⁸

i. Function

ALT catalyzes the reversible transfer of an amino groups in the L-alanine enzyme to α -ketoglutarate proteins forms such as pyruvate and L-glutamate.



ii. Clinical Significance

It is commonly estimated clinically as a parameter of diagnostic evaluation of hepatocellular injury in order to determine liver health. ALT has actually measured by international units/liter (IU/L) ^{9&10} when used in diagnosis. 10-40 IU/L are the standard reported range of experimental studies¹¹.

iii. Elevated levels

Significantly abnormal range of Alanine transaminase (ALT) often suggest the abnormality of conditions including viral hepatitis, diabetes mellitus induced cell necrosis, heart failure, liver injury, infectious

mononucleosis, bile duct problems and myopathy. Because of these reason, ALT is one of the important parameter used for screening of liver diseases. Dietary choline deficiency shows marked elevation in ALT levels. These enzyme variation levels of ALT do not have significance of that medical problem is present. Fluctuation of ALT level is normal during course of day and ALT levels can increase in response to strenuous physical exercise¹¹. When elevated ALT levels are found in blood concentration subsequently narrowed down by measuring other enzyme concentration (example liver- cell damage usually distinguished from biliary duct problems by measuring increased ALP). Myopathy-related ALT levels can be found out by measuring the creatine kinase enzymes. Several drugs elevate ALT levels, for example, Zileuton. For years, American Red Cross society used for ALT testing as part of the key enzyme of tests to ensure the safety of its blood pumping by deferring donors with elevated ALT levels. Main reason was to specify donors have an infection with Hepatitis C because there is a no specific test available for these¹².

1.1.4.2.Aspartate transaminase

Aspartate Transaminase (AST) also called Aspartate Aminotransferase (ASAT/AAT/AspAT) or Serum Glutamic Oxaloacetic Transaminase (SGOT), is a transaminase enzyme containing pyridoxal phosphate. AST catalyses reversible transfer of α -amino group between aspartate and glutamate .it is a key enzyme required for amino acid metabolism in human. It commonly present in liver, heart, skeletal muscle, kidneys, brain and red blood cells and AST is commonly measured clinically as a marker for liver health. It is also associated with liver parenchymal cell metabolism. The ratio of AST/ALT is may be useful for differentiation between etiology of liver damage ^{13, 14}. Reference range is 6-40IU/L ¹⁵.

i.Function

AST catalyzes the interconversion of aspartate and α -ketoglutarate to oxaloacetate and glutamate.

Aspartate (Asp) + α - Ketoglutarate \leftrightarrow Oxaloacetate + Glutamate (Glu)

As prototypical transaminase AST relies on PLP as a cofactor to transfer amino group from aspartate or glutamate to corresponding ketoacid¹⁶.

iii.Isoenzymes

Two isoenzymes are present in wide variety of eukaryotes. In humans,

- i. **GOT1 / c AST**, the cytosolic iso enzyme synthesized mainly from red blood cells and heart.
- ii. **GOT2 / m AST**, the mitochondrial isoenzyme present predominantly in liver.

These isoenzymes are considered to be evolved from a common gene duplication and subsequent synthesis¹⁷.

iv.Clinical significance:

It is raised in liver inflammation. It is also elevated in diseases such as myocardial infarction, acute pancreatitis, nephrotoxicity, hemotoxicity, musculoskeletal diseases and trauma. AST was used initially biochemical marker for diagnosis of acute myocardial infarction but now redundant and has been superseded by the cardiac troponins¹⁸. AST is commonly measured clinically as a part of diagnostic liver function test in order to determine liver health.

Reference range-

Male 8-40IU/L

Female 6-34IU/L

1.1.4.3.Alkaline phosphatase

Alkaline phosphatase (ALP) have functioning towards removing phosphate group containing molecules such as nucleotides, proteins, and alkaloids. Process of removing phosphate group is called de-phosphorylation. By the maintenance of effective and also alkaline environment. It is sometimes used

similarly as basic phosphatase¹⁹. ALP is mainly present in cells lining of the biliary ducts.

i.Elevated levels

ALP levels in plasma increase due to large bile duct constriction, intrahepatic cholestasis. ALP is found also in bone and placental tissue and hence higher in growing children and elderly patients haveing Paget's disease. In third trimester of pregnancy ALP is two to three times higher.

ii.Reference range: 30-120 IU/L²⁰.Liver (ALP): Cholestasis, cholecystitis, cholangitis, cirrhosis, hepatitis, fatty liver, sarcoidosis, liver tumor, liver metastases, drug intoxication²¹. Placental ALP is elevated in seminomas²² and active form of rickets as well as in following diseases²³.

- Biliary construction.
- Bone conditions.
- Osteoblastic bone cancer.
- Osteomalacia.
- Liver disorder/ hepatitis.
- Leukemia.
- Lymphoma.
- Paget's disease.
- Sarcoidosis.
- Hyperparathyroidism.

iii.Lowered levels

Following diseases may lead to decreased levels of alkaline phosphatase-

- Hypophosphatasia (autosomal recessive disease).
- Postmenopausal women undertaking estrogen therapy due to osteoporosis.
- Hypothyroidism or severe anemia.
- Children affected with achondroplasia and cretinism.
- Children who are victims of severe episode of enteritis.

-
- Pernicious and aplastic anemia.
 - myelogenous leukemia.
 - Wilson's disease.

Apart from these, the following chemicals are clinically studied to reduce alkaline phosphatase: Oral contraceptives²⁴.

1.1.4.4.Total protein

Total protein includes total amount of two classes of proteins present in fluid portion of blood. These include albumin and globulin. Total protein tests measures amount of albumin and globulin which are major groups of protein in blood. A low total protein level due to liver disorder, kidney disorder or disorder which protein is not digested or absorbed properly²⁵.

i.Normal Range: 6.0 - 8.3gm/dl

ii.Higher -than –normal levels may be due to:

- Chronic inflammation or infection (HIV, Hepatitis B or C).
- Bone marrow disorders (Multiple myeloma, Waldenstroms disease).

iii.Lower-than-normal levels may be due to:

- Bleeding (Hemorrhage).
- Burns (extensive).
- Liver disease.
- Glomerulonephritis and nephritic syndrome.
- Malabsorption.
- Malnutrition²⁶.

1.1.4.5.Total bilirubin

Total bilirubin (TBIL) test checks levels of bilirubin in blood. Bilirubin (orange-yellow pigment) is the waste product of normal break down of red blood cells. Bilirubin passes through liver and eventually passes out of body as feces and small amount in urine. Before reaching liver bilirubin is called

unconjugated. Inside liver it combines with certain sugars to create water soluble form called conjugated bilirubin²⁷.

i.Normal Range: 0.3 -1.9mg/dl

ii.Clinical significance

Total bilirubin is usually measured to screen for or to monitor liver or gallbladder diseases. Presence of high amount of bilirubin in body leads to jaundice.

iii.Higher Levels:

- Drug toxicity.
- Liver diseases such as hepatitis.
- Biliary stricture.
- Cancer of gallbladder or pancreas.
- Gallstones²⁸.
- Erythroblastosis fetalis.
- Physiological jaundice.
- Sick cell anemia²⁹.
- HIV Infection.
- Bacterial infection inside blood.

1.1.5.Hepatotoxicity

Liver diseases are the major medical problems faced by the people all over the world³⁰. About 20,000 deaths occur every year due to liver disorders³¹. In Africa and in Asia, the main causes of liver diseases are viruses and parasitic infections, whereas in Europe and in North America, a major cause is alcohol abuse³⁰. Liver diseases are mainly caused by toxic chemicals, excessive intake of alcohol, infections and autoimmune disorders³². Hepatotoxicity due to drug appears to be a common contributing factor. Liver is expected not only to carryout physiological functions but also to protect against the hazardous of harmful drugs and chemicals³³. Drug induced chemical injury is responsible for 5% of all hospital admissions and

50% of all acute liver failures. More than 75% of cases of immunological reaction of drugs leading to liver transplantation or death³⁴.

Hepatotoxicity mainly implies chemical-driven liver damage. Certain drugs when taken in overdose and sometimes even when administered within therapeutic ranges may injure many organs. Some chemical agents including those that are used in laboratories (Ccl₄, paracetamol) and industries (Lead, arsenic) and natural chemicals (microcystine, aflatoxins) and herbal remedies (cascara sagrada, ephedra) can also cause hepatotoxicity. Chemicals which cause liver injury are collectively known as hepatotoxins³⁴.

- NSAIDS³⁵ (Acetaminophen³⁶, Aspirin, Ibuprofen)
- Glucocorticoids.
- Anti-Tubercular drug (Isoniazid)³⁷.
- Industrial toxins (arsenic, carbon tetrachloride, vinyl chloride).
- Herbal remedies (Ackee fruit, camphor, cycasin, kava leaves, valerian, comfrey)³⁸.

1.1.5.1.Pathophysiological mechanisms

Pathophysiological mechanisms of hepatotoxicity are still being identified and which include both hepatocellular and extracellular effects. Following are some mechanisms:

Disruption of hepatocyte: Drugs can bind to intracellular proteins by covalent binding which result in a lower in ATP levels subsequent disruption. Splitting of actin these fibrils at the surface of the hepatocyte causes rupture of the membrane of liver.

Disruption of transport protein: Bile flow may be interrupted by drugs that affect transport proteins at canalicular membrane. Loss of villous due to the interruption of transport pumps leading to multidrug resistance- associated protein 3 prevent excretion of bilirubin resulting in cholestasis.

Cytolytic T-cell activation: Covalent binding of drug to P-450 enzyme acts as an immunogenic activation of T-cells and cytokines leading to immune reaction.

-
- **Apoptosis of hepatocytes:** Stimulation of these pathway may leading for programmed necrosis of hepatocytes.
 - **Mitochondrial disruption:** Some drugs inhibit mitochondrial function by dual effect on both beta- oxidation energy productions by inhibiting the release of the e dinucleotide, subsequently reduce the ATP production.
 - **Bile duct injury:** free radicle produced metabolites excreted in the bile may leading to necrosis of bile duct epithelium.

1.1.5.2. Drug toxicity mechanisms

Classic division of drug reactions is of at least 2 major groups which include:

- (1) Drugs which directly affect liver.
 - (2) Drugs which mediate an immune response.
-
- **Intrinsic / predictable drug reactions:** molecules that fall into this drug category lead to reproducible injuries in mammals and injury is related to dose. Injury can be due to drug itself or to metabolite. Acetaminophen is a suitable example of well-known predictable hepatotoxin at higher therapeutic doses. Another example is carbon tetrachloride.
 - **Idiosyncratic / unpredictable drug reactions:** These drug reactions can be subdivided into those that are classified as hypersensitivity or immunoallergic and those that are metabolic-idiosyncratic. It occurs without obvious dose-dependency and in an unpredictable fashion.³⁹

1.1.5.3. Symptoms

List of signs and symptoms depicted in various causes for Hepatotoxicity include 15 symptoms as listed below:

- Nausea
- Vomiting
- Abdominal pain
- Loss of appetite

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- Diarrhea
 - Tiredness
 - Weakness
 - Jaundice
 - Yellow eyes
 - Yellow skin
 - Enlarged liver
 - Abnormal liver function test results
 - Swelling in feet
 - Weight gain due to water retention
 - Prolonged bleeding time.

1.1.5.4. Treatment

These are various sources for hepatotoxicity treatment mode these selected by consultation by physician about the treatment or change in treatment regimen. Treatment of hepatotoxicity has dependent upon causative agent, degree of liver dysfunction and age and general health of patient. Treatments for hepatotoxicity include:

- Withdrawal of causative medication or removal from exposure to causative agent.
- Regular monitoring of patient and review of liver function – where liver dysfunction is mild to moderate and liver function is improving.
- Complete avoidance of alcohol and medication that may contribute to further liver damage.
- N-Acetylcysteine is used for paracetamol toxicity.
- Management of symptoms of liver damage.
 - ❖ Nutrition – with vitamin supplementation as required
 - ❖ Regular exercise in order to maintain muscle mass.
 - ❖ Ursodeoxycholic acid.

-
- Management of pruritus
 - ❖ Cholestyramine
 - ❖ Antihistamines.
 - Management of ascites
 - ❖ Low sodium diet.
 - ❖ Diuretics – furosemide, spironolactone.
 - ❖ Removal of fluid via a needle in the abdomen – Paracentesis.
 - ❖ Portosystemic shunting.
 - Management of portal hypertension
 - ❖ Beta - blockers
 - ❖ Oesophageal variceal banding
 - ❖ Portocaval shunt
 - Management of acute liver failure due to hepatotoxicity
 - ❖ Supportive care always in intensive care unit – airway protection, fluid and electrolyte management.
 - ❖ Management of complications such as bleeding problems and hepatic encephalopathy.
 - Liver transplantation – for acute fulminant liver failure or end stage cirrhosis.

1.1.5.5. Hepatotoxins

Agents which cause liver injury are called as hepatotoxins³⁴.

Table.No:1:Types of Hepatotoxic Agents⁴²

Types of Hepatotoxic Agents	
Inorganic agents	
Metals and metalloids:	Antimony, arsenic, beryllium, bismuth, boron, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, gold, phosphorous, selenium, tellurium, thallium, zinc.
Organic agents	
Natural plant toxins:	Albotocin, cycasin, icterogenin, indospicins, lantana, agaione, pyrrolizidines, safole, tannic acid.
Mycotoxins:	Aflatoxin, cyclochlorotine, ethanol, luteoskyrin, chratoxins, rubratoxins, sterignatocystins, griseofulvin, sporidesmin, tetracycline and other antibiotics.
Bacterial toxins:	Exotoxins (<i>C.diphtheriae</i> , <i>Cl.botulinum</i> , <i>Str.hemolyticus</i>), endotoxins, ethionine.
Synthetic Non-Medicinal:	Haloalkanes and haloolefins, nitroalkanes chloroaromatic compounds, nitroaromatic compounds, organic amines, azo compounds, phenol and derivatives, various other organic compounds.
Medicinal agents:	Over 100 drugs used for treatment and diagnosis.

I have selected paracetamol, ethanol and carbon tetrachloride to induce hepatotoxicity. Liver injury caused by hepatotoxins such as carbon tetrachloride, ethanol and acetaminophen is characterized by varying degrees of hepatocyte degeneration and cell death by either apoptosis or by necrosis. Generation or formation of reactive intermediate metabolites from metabolism of hepatotoxins and occurrence of reactive oxygen species (ROS) in inflammatory reaction accounts for variety of Pathophysiological pathways resulting in cell death includes covalent binding, tangled cytosolic calcium homeostasis, GSH depletion, starting of mitochondrial permeability transition (MPT) and associated lipid peroxidation⁴³.

a.Paracetamol induced hepatotoxicity

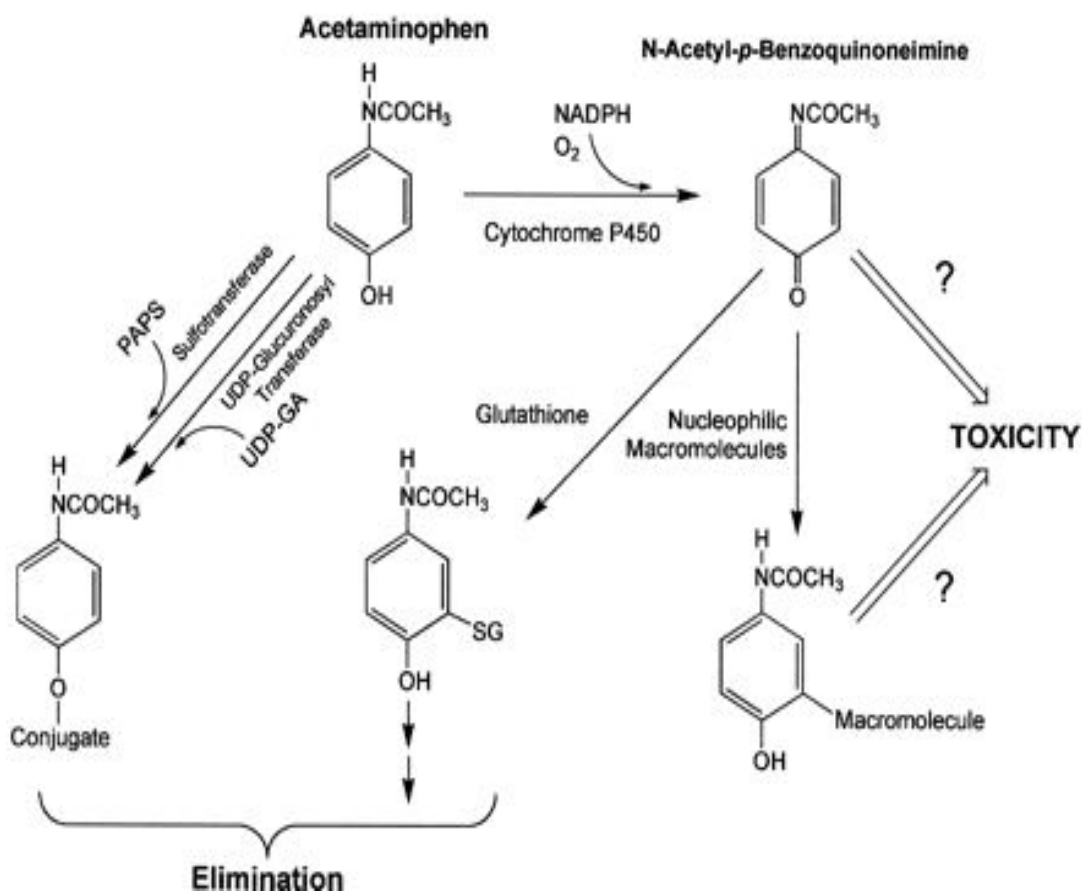
Paracetamol (Acetaminophen), a widely used analgesic and antipyretic drug that produces acute liver damage in high doses. Paracetamol related hepatotoxicity is now the most common cause of the potentially devastating clinical syndrome of acute liver failure in many western countries.⁴⁴ Most such instances are the consequence of ingestion of large paracetamol overdoses often taken at a single time point with suicidal or parasuicidal intent⁴⁵. Cases of severe hepatotoxicity high dose up to 10g daily or more cause other cytochrome P-450 enzyme inducing drugs⁴⁶.

Paracetamol induced hepatotoxicity is thought to be caused by N-acetyl-p-benzoquinoneimine (NAPQI), a cytochrome P-450 mediated intermediate metabolite⁴⁷. NAPQI can react with sulphhydryl groups such as glutathione and protein thiols. The covalent bonding of NAPQI to cell proteins is considered the initial step in a chain eventually leading to cell necrosis⁴⁸. It has been established that a hepatotoxic dose of paracetamol depletes endogenous glutathione level to below a threshold value (<20% of control), therefore permitting interaction of NAPQI with cell macromolecules⁴⁹.

i.Metabolic activation of acetaminophen

Acetaminophen causes potentially fatal hepatic centrilobular necrosis if taken in overdose. It is metabolically activated through cytochrome P-450 enzymes to reactive metabolite which depleted glutathione (GSH) and covalently bounds to protein. It shows that repletion of GSH prevented toxicity. Reactive metabolite was characterized to be N – acetyl – p – benzoquinone imine (NAPQI) ⁵⁰. After high dose ingestion of acetaminophen total hepatic GSH is decreased by as much as 90% and as a result metabolite covalently binds to the cysteins groups on proteins forming acetaminophen protein adducts. This mechanism is shown below,

Figure.No:5: Schematic Representation of Metabolic activation of Acetaminophen Toxicity⁵¹

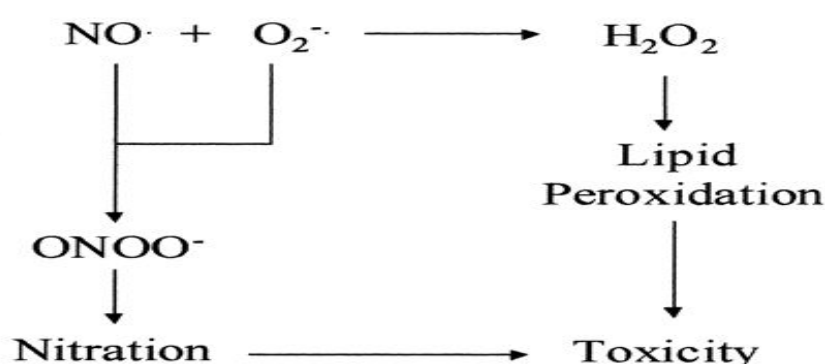


Events of hepatocellular necrosis subsequent formation of acetaminophen protein molecule adducts are not fully understood. One of the mechanism of cell death and formation of covalent bonding to critical cellular proteins lead to cell death and lysis. First major cellular targets have been initiated to formation of mitochondrial proteins with subsequent loss of energy synthesis as well as proteins involved in cellular ion control⁵². Blockage of mitochondrial ion concentration has to form a toxic mechanism involved in acetaminophen – mediated cell death. These ion losses can subsequently increases the cytosolic Ca^{2+} levels, lead to DNA strand breaks⁵³.

Oxidative stress is the one of important factor which will be responsible for the development of acetaminophen toxicity. Under conditions of NAPQI formation after toxic acetaminophen dose, GSH concentrations are very low in the centrilobular cells and major peroxide detoxification enzyme, GSH peroxidase was inhibited. When GSH levels are low, the metabolite fails to be

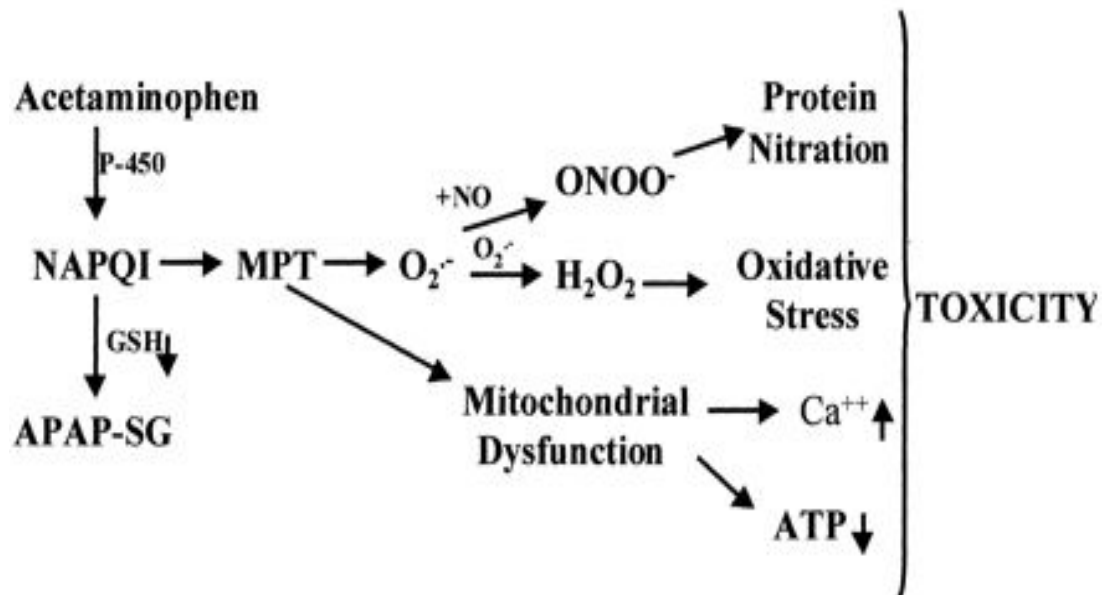
detoxified by conjugation; it accumulates and causes liver injury⁵⁴. Lipid peroxidation resulting from oxidative stress contributes to the initiation and recently reported data suggest that acetaminophen hepatotoxicity is mediated by an initial metabolic oxidation, covalent bonding and subsequent activation of macrophages to form reactive oxygen and nitrogen species⁵⁵. Protection against oxidation is provided by glutathione and by system of soluble and enzymatic defenses.

Figure. No: 6: Schematic Representation Depicting Role Of Oxidative Stress In Acetaminophen Toxicity⁵¹



Mitochondrial dysfunction is an important mechanism in acetaminophen induced toxicity. Mitochondrial permeability transition (MPT) occurs with the formation of superoxide and may be formation of superoxide radical may subsequent synthesis of peroxynitrite and tyrosine nitration. Oxidants like peroxides and peroxynitrite, Ca^{2+} and Pi promote onset of MPT, whereas Mg^{2+} , ADP, low PH and high membrane potential imbalance onset of action. Permeability change is associated with membrane depolarization, uncoupling of oxidative phosphorylation, releasing of intramitochondrial ions concentration, metabolic intermediates and mitochondrial oedema

Figure. No: 7 :Schematic Representation effect Of Mitochondrial Permeability Transition In Acetaminophen Toxicity⁵¹.



b.Ethanol induced hepatotoxicity

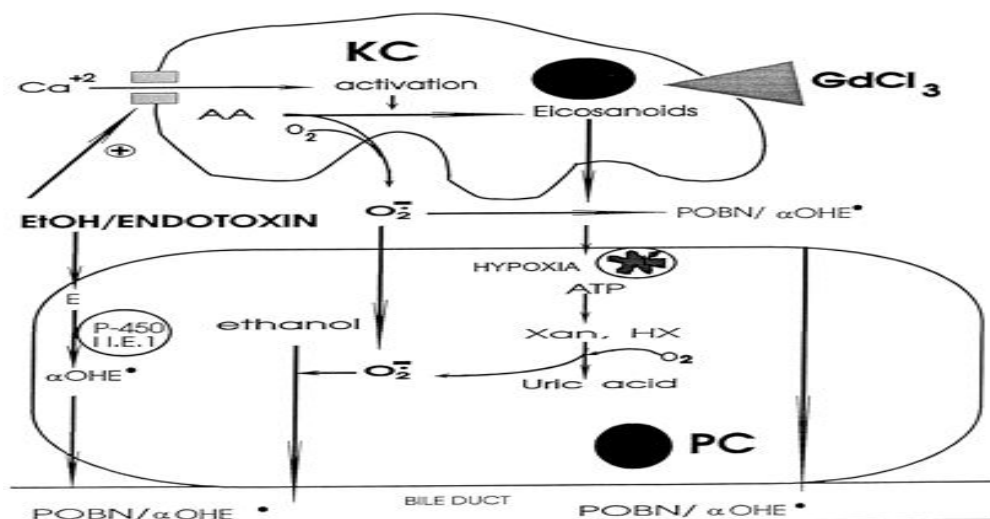
Ethanol induces number of deleterious metabolic changes in liver. Intake of ethanol for long time leads to development of steatosis, alcoholic hepatitis and cirrhosis resulting in weight and volume changes⁵⁶. About 80% of heavy drinkers had been reported to develop steatosis, 10-35% alcoholic hepatitis and approximately 10% liver cirrhosis⁵⁷.

i.Mechanism underlying Ethanol induced hepatotoxicity

Alcohol consumption results in increase in release of endotoxin from gut bacteria and membrane permeability of gut to endotoxin or both. Females are more often sensitive to these changes. Blood endotoxin is elevated and enters liver where it is engulfed by Kupffer cells that become activated releasing TNF- alpha, PGE2 and free radical. Prostaglandins increase oxygen uptake and are responsible for hypermetabolic state in liver. Increase in oxygen demand leads to hypoxia of liver and on reperfusion alpha - hydroxyethyl free radicals are formed that leads to tissue damage in oxygen poor pericentral regions of liver lobule. Blocking of these events can be done

by sterilization of gut using antibiotics or destruction of Kupffer cells with GdCl₃ and thus prevents liver injury⁵⁸.

Figure. No: 8: Mechanism of ethanol induced hepatotoxicity⁵⁹.



c.CCL₄ Induced hepatotoxicity

Administration of carbon tetrachloride to rodents is a commonly used model to investigate mechanism of hepatic damage. Reactive free radicals induced by carbon tetrachloride are initiative for cell damage by two different mechanisms which include (1) binding to membrane proteins followed by (2) lipid peroxidation⁶⁰. CCL₄ has high lipid solubility so well distributed in body but produces toxic effects largely to liver and kidneys. Toxicity increased by agents that induce microsomal drug metabolizing enzymes and reduced by inhibitors of microsomal enzymes.

i.Mechanism underlying CCL₄ induced hepatotoxicity

Microsomal mixed function oxidase system withdraws an electron from CCL₄ leaving reactive trichloromethyl radical (CCL₃^{*})⁶¹ by action of microsomal cytochrome P-450 enzyme (CYP2E11). This highly reactive free radical suddenly reacts with molecular oxygen to form trichloro methyl peroxy radical (CCL₃O₂). Both trichloro and peroxy radical can bind to cellular proteins and lipids initiating lipid peroxidation and liver damage⁶².

Trichloromethyl free radical has life time of only about 100 microseconds and so has time to diffuse for only short distance within liver cell before undergoing secondary reactions. Secondary reactions are responsible for biochemical damage may be of different kinds which include:

- a) Oxidation of thiols to form disulphide bonds.
- b) Saturation of double bonds in lipids, nucleotides, proteins which results in covalent attachment of free radical group of those sites.
- c) Lipid peroxidation reaction where polyunsaturated membrane lipids are converted to peroxide derivative and finally to aldehydes and other products leading to further cascade of reaction resulting in irreversible membrane damage.

Prolonged administration of CCl_4 leads to cirrhosis and hepatic carcinoma⁶¹.

1.1.6. Modern medicines for treatment of liver diseases

Liver diseases can be treated using allopathic as well as by using herbal drugs.

1.1.6.1. Hepatoprotective allopathic treatment

Few modern medicines are available for treating liver diseases that includes:

- 1) Ursodeoxycholic acid (Ursodiol)
Ursodiol decreases intestinal absorption and suppresses hepatic synthesis and storage of cholesterol. It is mainly used in management of chronic hepatic diseases in humans.
- 2) Penicillamine
Penicillamine chelates several metals like copper, iron, lead and mercury forming stable water soluble complexes which are renally excreted.

Other drugs:

Antiviral medication such as alpha interferon, ribavirin, steroids, antibiotics etc. are also used in liver diseases⁶³. Drugs like tricholinecitrates,

trithioparamethoxy phenyl propane, essential phospholipids, combination of drugs such as L-ornithine, L-aspartate and pancreatin, silymarin and Ursodeoxycholic acid are usually prescribed for hepatitis, cirrhosis and other liver diseases⁶⁴. N-acetylcysteine is used in early phases of acetaminophen toxicity. L-carnitine is potentially valuable during valproate toxicity. Cholestyramine can be used to alleviate pruritus³⁹.

i.Disadvantages of allopathic drugs

Side effects of many modern medicines are mostly alarming. Interactions, contra-interactions, side effects and toxicity of synthetic medicine vary from mild to severe that includes insomnia, vomiting, fatigue, dry mouth, diarrhea, constipation, dizziness, suicidal thought, depression, seizures, anemia, hair loss, high blood sugar, swelling, impotency, confusion, fainting and finally death⁶⁵. Antibiotics usually cause stomach upset or allergic reactions. Interferon shows side effects as flu-like illness with fever and body aches⁶³.

1.1.6.2.Herbal hepatoprotective drug treatment

A number of polyherbal preparations have been used in treating various liver disorders since ages. Some herbal formulations include:

- a) Liv-52: It is a non-toxic hepatoprotective drug from Himalaya Drug Co. Liv-52 can improve clinical parameters in patients having liver damage mainly in alcoholic liver damage.
- b) LIMARIN®: It has potent hepatoprotective and free radical scavenging (antioxidant) activity. It is derived from active extract of fruit of silybum marianum⁶³.

Some of the polyherbal formulations have been verified for hepatoprotective activity against chemical driven liver damage in experimental animals which include Liv52, Liv42, Jigrine, Koflet⁶⁶, Cirrhitin, Livex and Hepatomed etc.

1.1.6.2.1.Limitations of herbal preparations

Herbal- based preparations for treating liver disorders has been in use in India for long time and has been popularized worldwide by leading

pharmaceuticals. Despite of popularity of herbal medicines for liver diseases in particular, are still unacceptable treatment modalities for liver diseases. Limiting factors include:

- ❖ Lack of standardization procedures of herbal preparations.
- ❖ Lack of identification of active components and principles.
- ❖ Lack of randomized controlled clinical trials (RCTs).
- ❖ Lack of toxicological evaluation⁶⁷.
- ❖ Poor solubility.
- ❖ Poor bioavailability.
- ❖ Poor hepatic cell regeneration.

i..Hepatoprotective activity of silymarin

Mechanism of action of Silybin is complex and highly beneficial in protecting hepatocytes. It blocks penetration of various toxins into hepatocytes and thus prevents cell death. It protects liver from oxidative intracellular free radicals by increasing activity of enzyme superoxide dismutase and peroxidase as well as by increasing concentration of glutathione and activity of peroxidase. Silybin strengthens and stabilizes cell membranes, inhibits synthesis of prostaglandins associated with lipid peroxidation and promotes regeneration of liver through stimulation of protein synthesis and thus effects on production of new hepatocytes⁸⁸.Silybin acts in four different ways:

- ❖ Antioxidant, scavenger and regulator of intracellular content of glutathione.
- ❖ Cell membrane stabilizer and permeability regulator that prevent hepatotoxic agents from entering hepatocytes.
- ❖ Promoters of ribosomal RNA production, stimulating liver regulation.
- ❖ Inhibitors of transformation of stellate hepatocytes into myofibroblasts- process which is responsible for deposition of collagen fibers leading to cirrhosis⁸

CHAPTER II

2.LITERATURE REVIEW

Afiwa Missebukpo,et,al (2010) ⁹³ was investigated the hydro-alcoholic extract of *Ixora coccinea* (ICE) exhibit the anti-asthmatic activity in an ovalbumin (OVA) induced asthmatic rat model. These facts led us to examine their antioxidant activities. The free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity and the intracellularly antioxidant activity of ICE were determined. The protective effect of ICE against 2,2' azobis (2-amidinopropane) hydrochloride (AAPH)-induced red blood cell lysis was also evaluated. It was found that ICE could scavenge DPPH with an IC₅₀ of 283.3 µg/ml and protected red blood cell against AAPH-induced hemolysis with an IC₅₀ of 72.92 versus 52.08 µg/ml for ascorbic acid. Erythrocytes obtained from the ICE-administrated rats showed an enhanced resistance to hemolysis. In OVA-induced asthma, rats were sensitized and challenged with ovalbumin. The effect of ICE at 1500 mg/kg per os on malondialdehyde (MDA) production and lung catalase activity were determined. ICE significantly reduced the lipid peroxidation and enhanced catalase activity in lung ($p < 0.05$). In conclusion, the hydro-alcoholic extract of *I. coccinea* possesses an antioxidant activity and protective effect against free-radical-induced hemolysis

Prabu,et,al(2010) ⁹⁴ reported the anti-diarrhoeal activity of aqueous extract of the leaves of *Ixora coccinea* against a castor oil induced diarrhoea model in rats. The gastrointestinal transit rate was expressed as the percentage of the longest distance which was traversed by the charcoal, divided by the total length of the small intestine. The weight and the volume of the intestinal content induced by castor oil were studied by the enteropooling method. Loperamide was used as a positive control. The plant-extract showed significant ($P < 0.001$) inhibitor activity against castor oil induced diarrhoea and castor oil induced enteropooling in rats at the dose of 400 mg/kg. There was significant reduction in gastrointestinal motility by the charcoal meal test in rats.

Moni Rani,et,al (2008)⁹⁵ reported the anti-oxidant activity of the methanol extract of *Ixora coccinea* L by DPPH free radical scavenging assay, reducing power and total antioxidant capacity using phosphor molybdenum method. Preliminary phytochemical screening revealed that the extract of the leaf of *Ixora coccinea* possesses flavonoids, steroids and tannin materials. The methanolic extract showed significant activities in all antioxidant assays compared to the standard antioxidant in a dose dependent manner and remarkable activities to scavenge reactive oxygen species (ROS) may be attributed to the high amount of hydrophilic phenolics. In DPPH radical scavenging assay the IC₅₀ value of the extract was found to be 100.53 µg/mL while ascorbic acid had the IC₅₀ value 58.92 µg/mL. Thus *Ixora coccinea* extract showed strong reducing power and total antioxidant capacity.

Latha,et,al (2010)⁹⁶ reported the hepatoprotective activity in ethanolic extracts of three different plants *Ixora coccinea* (IC), *Rhinacanthus nasuta* (RN), *Spilanthes ciliata* (SC) on the aflatoxin B1 (AFB1) –intoxicated livers of albino male Wistar rats. Biochemical parameters, including serum hepatic enzymes (glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and alkaline phosphatase), were studied. Pre-treatment of the rats with oral administration of these plant ethanolic extracts, prior to AFB1 was found to provide significant protection against toxin induced liver damage, determined 72 hours after the AFB1 challenge (1.5 mg/kg, intraperitoneally) was evidenced by a significant lowering of the activity of the serum enzymes and enhanced hepatic reduced GSH status. Pathological examination of the liver tissues supported the biochemical findings. The three plant extracts, IC, RN and SC, showed significant anti-lipid peroxidant effects *in vitro*.

Nagaraj,et,al (2011)⁹⁷ reported the synthesis of gold nanoparticles in aqueous medium using leaf extracts of *Ixora coccinea* as reducing and stabilizing agent. On treating chloroauric acid solution with extract, rapid reduction of chloroaurate ions is observed leading to the formation of the highly stable gold nanoparticles in solution. The synthesized nanoparticles are confirmed by colour changes and it has been characterized by UV-visible spectroscopy. Presence of this strong broad plasmon peak has been well documented for various Me- NPs, with sizes ranging all the way from 2 to 100

nm. The morphology and size of the biologically synthesized gold nanoparticles were determined using TEM. The images clearly showed that the average size of the nanotriangles is about 200 nm, while, the spherical like particles show very small size about 5-10 nm. This study also showed that gold nanoparticles with antibiotic show more inhibitory zones than compared to the standard antibiotics.

Panikar,et,al(1998)⁹⁸ reported the antitumour activity of *Ixora coccinea* L. (Rubiaceae) leaf was studied in comparison to intraperitoneally transplanted Dalton's lymphoma (ascitic and solid tumours) and Ehrlich ascites carcinoma (EAC) tumours in mice. Intraperitoneal administration of 200 mg/kg of the active fraction (AF) of the *I. coccinea* leaf increased the life-span of DLA and EAC ascitic tumour-bearing mice by 113 and 68%, respectively. The AF showed less activity against solid tumours (DLA) as compared to ascitic tumours. The same active fraction showed 50% cytotoxicity to DLA, EAC and Sarcoma-180 (S- 180) cells in vitro at concentrations of 18, 60 and 25 µg/ml, respectively. It was not toxic to normal lymphocytes, whereas it was toxic to transformed lymphocytes from leukaemic patients, acute lymphoblastic leukaemia (ALL) and chronic myelogenous leukaemia (CML) and K-562 suspension cell cultures. The AF inhibited tritiated thymidine incorporation in cellular DNA. Thus the anti-tumor activity of *Ixora coccinea* plant was proved.

Yasmeen,et,al(2011)⁹⁹ reported the hypoglycaemic and the hypolipidaemic activity of the aqueous extract of the leaves of *Ixora Coccinea* Linn in alloxan induced diabetic albino rats. The aqueous extract of leaves of *Ixora Coccinea* showed significant reduction ($p < 0.01$) in the blood glucose levels and the serum lipid profile levels, with 400 mg/kg of body weight in the alloxan induced diabetic rats as compared to the controls.

Elumalai,et,al(2012)¹⁰⁰ was studied the phytochemical and ethano pharmacological profile of *Ixora coccinea* and he reported it have anti-oxidant, anti-inflammatory and antidiabetic activity.

CHAPTER III

3. AIM AND OBJECTIVES

Plants that cure liver diseases so considerable interest has developed in the examination of these numerous plants remedies which are useful in liver diseases. So it is necessary to find new drugs of importance in hepato protective activity with fewer side effects. Moreover it is necessary to produce scientific validation to drugs of herbal origin in common use under Ayurvedic Siddha Unani systems of medicine. Why I have to select this particular disease is few effective drugs available for modern therapy, it produce side effect during the treatment is worse than the condition of liver damage

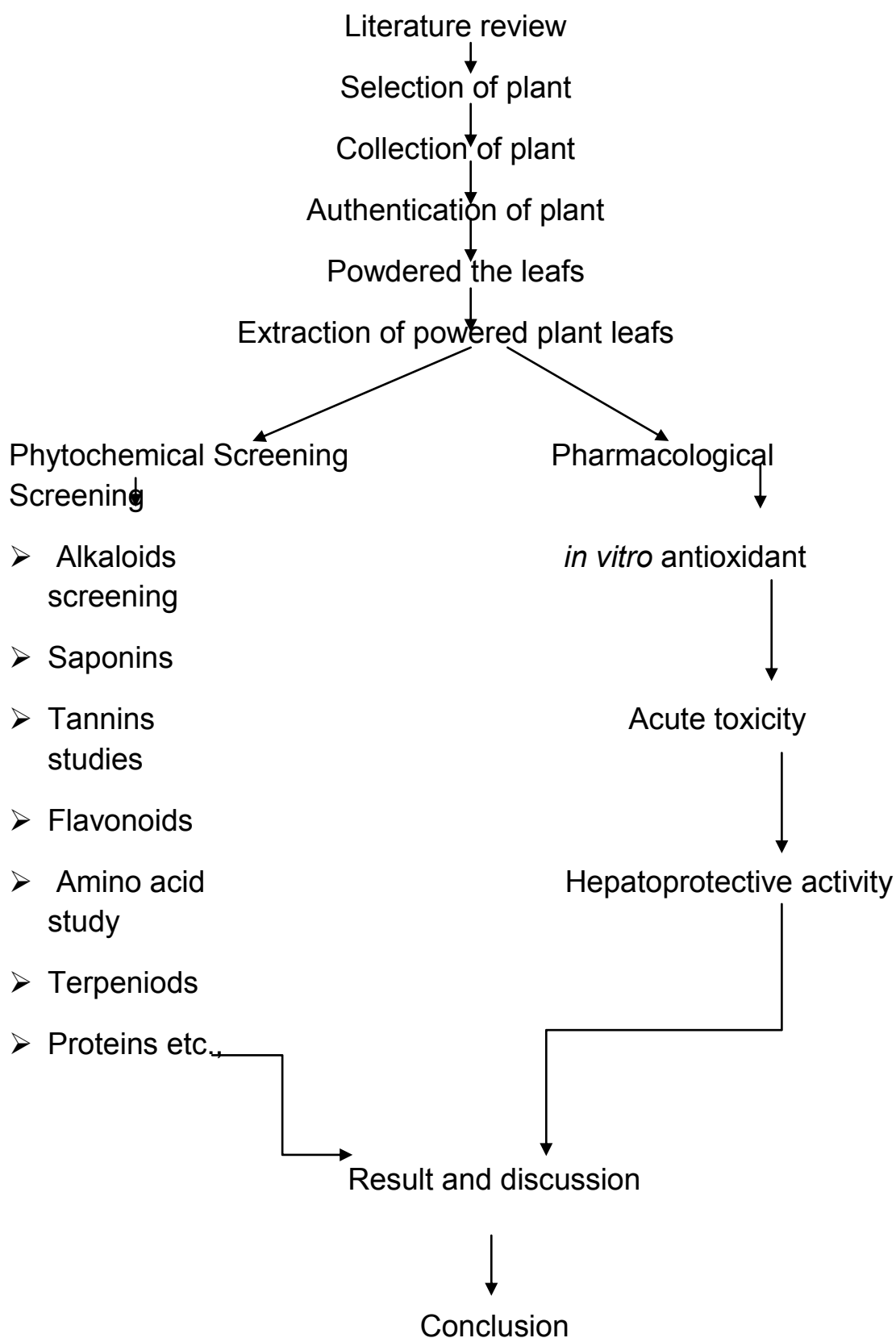
Phytochemical investigation will be a useful tool for the identification and authentication of the plant for industrial and further research purpose. Total phenol content of a tested material is related to the antioxidant activity. Antioxidants, which can scavenge free radicals, have an important role in pharmacological systems. Antioxidants are emerging as prophylactic and therapeutic agents. Hence, antioxidant was also evaluated for the potent extract.

And now I have undertaken the study of evaluation of anti-oxidant and hepatoprotective activity of ixorea coccinea leaf extracts by various hepatotoxin induced albino rat models

- To select plant based on their ethno medical uses and preparation of their extracts.
- To screen phytochemical profile.
- To screen the selected extract for antioxidant using various in vitro methods
- To screen the potent plant extract for their in vivo hepatoprotective activities

CHAPTER IV

4. PLAN OF WORK



CHAPTER V

5.PLANT PROFILE

*IXORA COCCINEA*¹⁰¹

Figure. No:9: *ixora coccinea* palnt



5.1. Taxonomical classification¹⁰²

Table.No:2:Taxonomical classification of *Ixora coccinea*

Kingdom	Plantae
Subkingdom	<u>Tracheobionta</u>
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	<u>Asteridae</u>
Order	<u>Rubiales</u>
Family	<u>Rubiaceae</u>
Genus	<i>Ixora</i>
Species	Coccinea

5.2.Vernacular names of *Ixora coccinea*¹⁰³

Table.No:3:Vernacular names of *Ixora coccinea*¹⁰³

English	<i>Ixora</i>
Hindi	<i>Rugmini</i>
Tamil	<i>Vedchi</i>
Bengali	<i>Rangan</i>
Malayalam	<i>Chethi</i>

5.3.Distribution and habitat¹⁰⁴

Ixora coccinea is a common leafing shrub native to Southern India and Sri Lanka and widely cultivated in Indonesia, Malaysia, the Philippines, Vietnam, Cambodia, Laos and Thailand. It has become one of the most popular leafing shrubs in South Florida – USA gardens and landscapes. It grows in tropical areas with in medium annual rainfall in well drained soils.

5.4.Description

Ixora coccinea is a low-growing tropical shrub notable for its bright coloured leafs which are composed of many small blooms massed together into dense, flat-topped leaf heads. *Ixora coccinea* is one of the few *Ixora* species that make good indoor plants along with several kinds developed from it.

It takes up to five years for *Ixora coccinea* to grow to its maximum height of about 1.2m (4 feet). It is a much-branching shrub, with leathery, shiny, pointed oblong leaves up to 10cm (4 inch) long and 5cm (2 inch) wide arranged in pairs or whorls of three or more in 1-2cm (0.4-0.8 inch) long stalks. Leaf color is dark green, often bronzish when the leaves are new. Tubular leafs, which are up to 5cm (2 inch) long and fiery red, open at the month into four petals arranged in the form of a cross about a centimeter (0.4 inch) wide. The entire leaf head has a diameter of 8-12cm (3-5 inch). Normal leafing period is summer, but occasional leafs also appear in the autumn.

Various kind of *Ixora* which have *Ixora coccinea* as a parent produce differently coloured blooms, chiefly in shade of orange, yellow and pink, as well as red.

5.5.Propagation

Propagate *Ixora coccinea* from stem cuttings 5-8cm (2-3 inch) long taken in spring. Trim each cutting immediately below a leaf, remove that leaf and dip the cut end in hormone rooting powder. Plant the cutting in a 5-8cm (2-3 inch) pot containing a moistened equal-parts mixture of peat moss and coarse sand or perlite. Enclose the whole in a plastic bag or propagating case and stand it in bright filtered light at a temperature of 21-27°C (70-81°F). When the cutting has rooted – probably in four to six weeks – uncover it gradually over a two or three week's period in order to acclimatize the new plant to the less humid atmosphere of the room. When the new plant is fully uncovered, begin to water moderately (allowing a couple of centimeters (0.4-0.8 inch) or so of the potting mixture to dry out between watering again) and apply standard liquid fertilizer once every two weeks. About three months after the start of the propagation move the new plant into a slightly bigger pot of the recommended potting mixture for adult plants and treat it as mature.

5.6.Uses¹⁰⁵

It is primarily ornamental plant. Cut specimens are long lasting and are often used in floral arrangements. It is sometimes used as a hedge. In tropical Asia the leafs, bark and leaves of this plant are used in traditional medicine.

CHAPTER VI

6. MATERIALS AND METHODS

6.1. Collection and authentication of plant

Ixora coccinea leaf was procured from the Botany Central council for Research in Ayurveda and Siddha Govt of India. (Certificate No:UWAU/350/16) The dried leafs are authenticated by Chelladurai.V research officer Botany Central council for Research in Ayurved and Siddha Govt of India.

6.2. Extraction Procedure:

i. Preparation of *Ixora coccinea* leaf extract¹⁰⁶

The leaf were initially separated from the main plants body and rinsed with distilled water and shade dried and then homogenized into fine powder and stored in air tight bottles. A total of 10 g of leaf air dried powder was weighed and was placed in 100 mL of organic solvents (methanol and ethanol) in a conical flask and then kept in a rotary shaker at 190-220 rpm for 24 h. And then it was filtered with the help of muslin cloth and centrifuged at 10 000 rpm for 5 min. The supernatant was collected and the solvent was evaporated by solvent distillation apparatus to make the final volume of one-fourth of the original volume, giving a concentration of 40 mg/mL. It was stored at 40 °C in air tight bottles for further studies.

6.3. Phyto chemical screening¹⁰⁷⁻¹⁰⁸

The plant may be containing the following compound such as carbohydrate, protein, and lipids. That is utilized as food by man. It also contains the compound like. Tannins, glycosides, alkaloids. Volatiles oils. The compound that is responsible for lots of medicinal properties

6.3.1.Test for carbohydrates

6.3.1.1..Molisch test

The sample powdered was added with 1 ml of alpha naphthol solution along with conc Sulphuric acid solution in the test tube reddish colour was produced at the junction between 2 liquid

6.3.1.2..Fehling test.

To the sample powder was added with both Fehling A and Fehling B solution and placed in the water bath for a sufficient time. This shows the brick red colour.

6.3.1.3..Benedicts test.

To the sample powder add 8 drops of benedict's reagents and Boil the sample vigorously for 5 min it shows the red ppt.

6.3.2.Test for alkaloids

To the small of stored powder (sample) was taken and add few drops of hydrochloric acid and filtered. The filtered was tested with various alkaloid agents.

6.3.2.1.Mayer's reagents

To a small of above filter add small quantity of Mayer's reagent to form cream precipitate.

6.3.2.2..Dragendorff's reagents

From the above filter add small amount of Dragendorffs reagents it forms a orange brown precipitate.

6.3.3.Test for flavonoids

To the filter of the plant extract add 5 ml of dilute ammonia solution and followed by the addition of concentrated sulphuric acid. It forms a yellow colour.

6.3.4.Test for steroids.

6.3.4.1.Salkowski test

Few amount of plant extract was mixed with chloroform and the same volume of sulphuric acid is added on it. Cherry red colour was obtained in the chloroform layer.

6.3.4.2.Libbbermann burchatd test:

The extract is dissolved in 2 ml of chloroform 10 drops of acetic acid and conc. Sulphuric acid were added. Now the solution becomes reddish colour then it turns to bluish green colour.

6.3.5.Test for tannins.

From few amount of plant extract is treated with vanillin hydrochloric acid reagent. It forms, pink or red colour due to the formation of phloroglucinol,

6.3.6.Test for protein.

6.3.6.1..Millon's reagents.

Millon's reagents (mercuric nitrate in nitric acid containing a trace of nitrous acid) usually yields a white precipitate on addition to a protein solution which turns red on heating.

6.3.6.2.Ninhydrin Test.

From the sample solution add 2 drops a freshly prepared 0.2% ninhydrine reagent was added to the extract and heating. Development of blue colour may indicate the presence of peptide, amino acid (PROTEIN).

6.3.7.Test for glycosides

6.3.7.1.Keller- killani test.

From the small quantity of small powder acetic acid was dissolved and adds few drops of ferric chloride and transferred to the

surface of conc Sulphuric acid. At the junction, reddish brown colour was formed,

6.3.8.Test for saponins.

6.3.8.1.Foam test

1 ml of extract solution is diluted separately with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. A 1 cm layer of foam indicates the presence of Saponins.

6.3.9.Test for Terpenoids:

About 0.5 g of plant extract in separate test tube was taken with 2 ml of chloroform; 5 ml of concentrated sulphuric acid was carefully added to form a layer and observed for presence of reddish brown color interface to show positive results for the presence of terpenoid.

6.4. *In vitro* antioxidant activities

6.4.1. Superoxide radical scavenging activity¹⁰⁹

Principle:

The superoxide anion radical scavenging activity was determined by nitro blue tetrazolium (NBT) reduction method of Mc Cord and Fridovich (1969). The assay is based on the ability of drug to inhibit the reduction of nitro blue tetrazolium (NBT) by Superoxide, which is generated by the reaction of photo reduction of riboflavin within the system. The superoxide radical thus generated reduce the NBT to a blue colored complex.

i.Reagents

- Nitro blue tetrazolium (NBT) - 1.5nm (12.3mg/10ml)
- Riboflavin - 0.12µm (4.5mg/100ml)
- NaCN/EDTA - 0.0015% NaCN in 0.1M EDTA
- Phosphate buffer - 0.06M (pH 7.8)

ii.Procedure:

The reaction mixture contained EDTA (0.1 M), 0.3mM NaCN, Riboflavin (0.12mM), NBT (1.5 n moles), Phosphate buffer (67mM, pH 7.8) and various concentrations of the seed oil extract in a final volume of 3ml. The tubes were illuminated under incandescent lamp for 15min. The optical density at 560 nm was measured before and after illumination. The inhibition of superoxide radical generation was determined by comparing the absorbance values of the control with that of seed oil extract and fraction-IV. Vitamin C was used as positive control. The concentration of fraction-IV required to scavenge 50% superoxide anion (IC₅₀ value) was then calculated.

iii.calculation

$$\% \text{ inhibition} = \frac{OD \text{ of control} - OD \text{ of sample}}{OD \text{ of control}} \times 100$$

6.4.2. DPPH radical reducing activity ¹¹⁰:

i.Principle

It is a rapid and simple method to measure antioxidant capacity. It involves the use of free radical, DPPH (2, 2- Diphenyl - 1- picryl hydrazyl) (Aquino et al, 2001). The odd electron in the DPPH free radical gives a strong absorption maximum at 517nm and is purple in color. The color turns from purple to yellow when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolourisation is stoichiometric with respect to the number of electrons captured.

i.reagent

- DPPH - 3mg in 25ml methanol (stored in dark bottle)
- Methanol

ii.Procedure

Freshly prepared DPPH (187 µl) was taken in different test tubes protected from sunlight. To this solution added different concentrations (0, 25,

50, 75,100,150,200µg/ml) of seed oil extract and fraction-IV. The volume was made up to 1ml with methanol. Keep the tubes in dark and after 20 min absorbance was measured at 515nm. Methanol was used as blank and vitamin C was used as positive control. The concentration of test materials to scavenge 50% DPPH radical (IC₅₀ value) was calculated from the graph plotted with % inhibition against Concentration.

iii.Calculation

$$\% \text{ inhibition} = \frac{OD \text{ of control} - OD \text{ of sample}}{OD \text{ of control}} \times 100$$

6.5. Pharmacological screening

6.5.1. Acute toxicity study¹¹¹

Experimental Protocol

Guideline	:	OECD – 423
CPCSEA Reg. No	:	KU/IAEC/M.pharm/172
Test	:	Limit test
Species	:	<i>Rattus norvegicus</i>
Strain	:	Albino Wistar rats
Number of animals	:	05
Sex	:	Male/female
Initial dose	:	5mg/kg
Route of administration	:	Oral
Duration observation	:	3hr close observation, followed by 14 days observation
Others	:	Body weight, water intake, mortality status
Parameters	:	CNS, ANS and behavioral changes
Blood collection	:	Not needed
Sacrifice	:	On day 14 after oral administration

Table.No:4:Experimental designs

Group	Dose(mg/kg)
Group I	5
Group II	50
Group III	300
Group IV	2000

i.Study design

Test animal – 6-8weeks old Adult Wistar rats of male and female, nulliparous and non-pregnant animals were obtained from centralized animal house from lab and acclimatized to holding for 1 week prior dosing.

ii.Housing conditions

Temperature – The experimental animal room temperature maintained at 22°C±3°C OECD guideline-423, 2001. These ranges are designed to allow homeotherms to maintain metabolic rate or to be within their thermo neutral zones. Because, temperature below the recommended range leads to increased food intake, increased energy expenditure but decrease in efficiency. In contrast, temperature above the recommended range leads to decreased food intake, decreased weight and decreased energy expenditure. Toxicity can vary with temperature might increase with linearity with temperature.

Humidity – The relative humidity maintained at 40%-60% preferably not exceeds 70% (OECD-423, 2001).The relative humidity below the recommended range can develop lesions such as ring tail and food consumption may be increased.

Light – 12-12 hours, Light/dark cycle. Appropriate lighting and light cycle play a key role in maintaining the physiology and the behavior rat. Light provided

for adequate vision and for neuroendocrine regulation of diurnal and circadian cycles (CPCSEA guidelines for laboratory animal facility 2003).

Light intensity – The light intensity maintained at 325 lux approximately 1m above the floor. Consideration of variations in light intensity, for the arrangement of animals on cage rack for toxicology study is necessary.

Caging – Polypropylene cages with solid bottom and walls. Lids made up of stainless steel grill capable of holding of both feed and water.

ii.Feeding condition – Sterile laboratory feed (*ad libitum*) and RO water bottles daily.

iii.Feed – Brown colored chow diet

iv.Drug administration – Animals were fasted for 12hour prior to dosing on day 0. Treatment rats were dosed by oral gavages, using a curved and ball tipped stainless steel feeding needle, with 20% gum acacia solution.

v.Clinical observations – All rats were monitored continuously for 4 hour after dosing for signs of toxicity. For the remainder of the 14 days study period, animals were monitored and any additional behavioral or clinical signs of toxicity. Animal's body weight was measured prior to dosing and on days 7 and 14. On all animals were killed and at the end of the study LD₅₀ value was established. Clinical observations and gross pathological examination was carried out.

6.5.2. Evaluation of hepatoprotective activity

6.5.2.1.Paracetamol induced liver toxicity

i.Animals

Healthy adult male Albino rats (120-200 gram body weight) were procured and randomly assigned to 5 groups, each containing 6 animals in polypropylene cages layered with husk and maintained in a controlled room at a temperature (22±3°C) and light (12 hours light/dark cycle). Animals were allowed free access to water and standard pellet diet. Animals were cared in accordance with the "Guide for the care and use of laboratory animals" and

study was conducted in accordance with CPCSEA. All animal experiments were conducted during the present study got prior permission from Institutional Animal Ethics Committee (IAEC approved) and following the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division, Government of India (No:- IAE 1012/c/07/ CPCSEA-Corres-2013-

ii.Methodology¹¹²:

The paracetamol suspension was freshly prepared at a dose of 2 gm/kg of paracetamol in 1ml of distilled water¹¹¹. Animals group received a single dose of paracetamol 2gm /kg through oral route for 7 day. Animals were allowed to develop hepatotoxicity, which was identified by biomarkers. Hepatotoxicity induced animals were selected for 28 days treatment. (Table 2)

Table.No:5:Experimental design of paracetamol induced liver toxicity

Group	Treatment
1	2gm/kg of Paracetamol in 1ml of distilled water to induce hepatotoxicity and received no other treatment which is served as paracetamol control.
2	2gm/kg of Paracetamol in 1ml of distilled water. After hepatotoxin induction, animals were treated orally with pure silymarin(100 mg/kg of body weight)
3	2gm/kg of Paracetamol in 1ml of distilled water. After hepatotoxin induction, animals were treated orally with prepared extract (100 mg/kg of body weight)
4	2gm/kg of Paracetamol in 1ml of distilled water. After hepatotoxin induction, animals were treated orally with prepared extract (200 mg/kg of body weight)
5	2gm/kg of Paracetamol in 1ml of distilled water. After hepatotoxin induction, animals were treated orally with prepared extract (400 mg/kg of body weight)

6.5.2.2.Evaluation of hepatoprotective efficacy:

Efficacy of the extract was assessed using biochemical marker enzymes such as,

- SGPT.
- SGOT.
- ALP (Alkaline Phosphatase).
- Total Protein.
- Total Bilirubin.
- Change in body weight.

6.5.2.3.Sample collection and biochemical assay:

The blood samples obtained were collected into plain sample tubes and centrifuged at 2000 rpm for 5 minutes to separate serum. Serum was carefully collected and kept in eppendorf tubes for the determination of the biochemical parameters.

6.5.2.4.Assessment of serum marker enzymes:

Serum level of SGPT and SGOT: These are the potential biomarkers of hepatic injury and were estimated using a commercial kit (Medsources Ozone Biomedicals Pvt. Ltd) (Reitman's and Frankel, 1957). Serum total protein (T. Protein) was determined using the method Lowry et al 1951). Serum total bilirubin (T. BIL) was determined using the method (Mallay et al 1937). Alkaline phosphatase (ALP) was determined using the method (King et al 1954), were assayed using standard Diagnostic kits at Benghazi medical center.

6.5.2.5.Change in Body Weight:

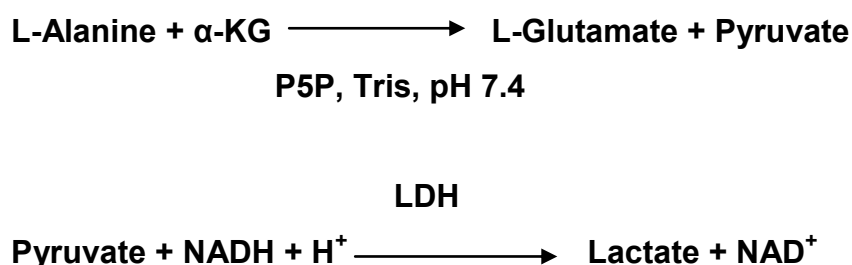
Animal body weight was measured weekly once from the starting day to end of the study in paracetamol, CCl_4 and ethanol induced models.

6.5.2.6.Serum enzyme parameters

a.Alanine transaminase (ALT)¹¹³

i.Principle of Procedure

ALT catalyzes the transamination of L-Alanine to α -Ketoglutarate (α -KG), forming L-Glutamate and Pyruvate. The Pyruvate formed is reduced to Lactate by Lactate Dehydrogenase (LDH) with simultaneous oxidation of reduced Nicotinamide-Adenine Dinucleotide (NADH). The change in absorbance is measured using a dichromatic (340, 700 nm) rate technique.



ii.Summary

The ALT method is an adaptation of the recommended procedure of the IFCC as described by Bergmeyer¹¹⁸. The procedure is based on the principles outlined by Wroblewski and LaDue but is modified to contain pyridoxal-5-phosphate (P5P) as an activator and to replace phosphate buffer with tris (hydroxymethyl) amino methane. Quantitation of ALT is a useful parameter in evaluating liver function. Significant elevations of ALT are found in diseases of the liver, such as hepatitis, necrosis, jaundice and cirrhosis. ALT levels can be elevated even before clinical jaundice appears.

b.Aspartate transaminase (AST)

i.Principle of Procedure¹¹⁴

AST catalyzes the transamination from L-Aspartate to α -Ketoglutarate, forming L-Glutamate and Oxaloacetate. The Oxaloacetate formed is reduced to malate by Malate Dehydrogenase (MDH) with simultaneous oxidation of reduced Nicotinamide Adenine Dinucleotide (NADH). The change in absorbance with time due to the conversion of NADH

to NAD is directly proportional to the AST activity and is measured using a bichromatic (340, 700 nm) rate technique.



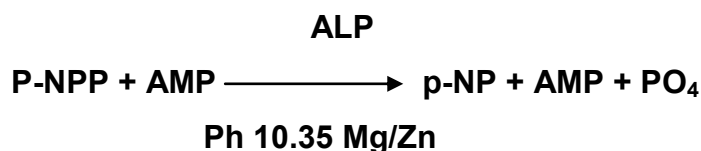
ii.Summary

The AST method is an adaptation of the methodology recommended by the International Federation of Clinical Chemistry (IFCC). The method uses the coenzyme Pyridoxal-5-Phosphate (P5P) to activate the apoenzyme and lactic acid Dehydrogenase (LDH) to eliminate pyruvate interference. AST levels are 10-200 fold elevated in patients with acute hepatic necrosis, viral hepatitis and drug induced poisoning. SGOT levels are also elevated 10 fold in patients with post hepatic jaundice, intra hepatic cholestasis and less than 10 fold in alcoholic and hepatic steatosis.

c.Alkaline phosphatase (ALP)

i.Principle of Procedure¹¹⁵

ALP catalyzes the transphosphorylation of p-nitrophenylphosphate (p-NPP) to p-nitro phenol (p-NP) in the presence of the transphosphorylating buffer, 2-amino-2-methyl-1-propanol (AMP). The reaction is enhanced through the use of magnesium and zinc ions. The change in absorbance at 405 nm due to the formation of p-NP is directly proportional to the ALP activity, since other reactants are present in non-rate limiting quantities and is measured using a bichromatic (405, 510 nm) rate technique.



ii.Summary

Measurements of ALP are used in the investigation of hepatobiliary and bone disease¹¹⁹. The ALP method is based on a procedure published by Bowers and McComb and more recently reviewed by Rej. This method responds to all ALP isoenzymes in human serum. Marked increase of ALP is seen in obstructive jaundice and biliary cirrhosis.

6.5.8.Histopathology Study¹¹⁶

- Rats from all the treatment groups and control groups were euthanized on the day 7. After gross observation, liver was collected and fixed in 10% Neutral Buffer Formalin.
- Trimming: Tissues were trimmed from all the lobes of liver.
- Processing: Processing is done with the help of Automated Tissue Processor (ATP) (Leica ASP 300) for 16 hours.
- Embedding: Processed tissues were embedded in paraffin with the help of paraffin embedding station (Leica EG 1150 H).
- Sectioning: Initially blocks were trimmed at 25 microns and then sectioned at 4 microns with the help of semi-automatic Microtome (Leica RM 2245).
- Staining: Slides were stained by H&E stain at Multistainer (Leica ST 5020).
- All the H&E stained slides were observed for pathological findings.

6.5.9.Statistical analysis:

N = 6, Values are Mean±SEM calculated using One way ANOVA followed by Tukey's multiple comparison test, *P<0.05 and **P<0.001 compared with Toxic treated group; **P<0.05 compared with vehicle treated group; # P<0.01.

CHAPTERVII

7. RESULTS AND CONCLUSION

7.1.Soxhlet Extraction of *Ixora coccinea* leaf Extract (ICLE)

The percentage yield of the ICLE in ethanol by cold maceration was found to be 2.93%w/w.

Table.No:6:Extraction of *Ixora coccinea* leaf Extract (ICLE)

Plant	Part used	Method of Extraction	Solvents	Percentage Yield (%W/V)
<i>Ixora coccinea</i>	leaf	Maceration	Ethanol (95%)	2.93

7.2.Preliminary phyto chemical screening.

Ixora coccinea leaf extracts (ICLE) was subjected various chemical tested as per the standard methods for the identification of the various constituents if this phyto chemical analysis is listed below.

7.2.1Test for carbohydrates

i.Molisch test

No reddish colour was produced at the junction between 2 liquid it indicate the absence of carbohydrates

ii.Fehling test.

This shows the blue colour. It indicate the absence of carbohydrates

iii.Benedicts test.

shows the yellow ppt. It indicate the absence of carbohydrates

7.2.2.Test for alkaloids

i..Mayer's reagents

It form cream precipitate. It indicate the absence of alkaloid

ii.Dragendorff's reagents

It forms a orange brown precipitate. It indicate the absence of alkaloid

7.2..3.Test for flavonoids

It forms a yellow colour. It indicates the presence of flavanoid

7.2.4.Test for steroids.

i.Salkowski test

Cherry red colour was obtained in the chloroform layer. It indicate the absence of steroid

ii.Libbbermann burchatd test:

solution becomes reddish colour then it turns to bluish green colour. It indicate the absence of steroid

7.2.5.Test for tannins.

It forms, pink or red colour due to the formation of phloroglucinol, It indicate the absence of tannins

7.2.6.Test for protein.

I.Millon's reagents.

solution which turns red on heating. It indicates the presence of protiens

II.Ninhydrin Test.

Development of blue colour may indicate the presence of peptide, amino acid (PROTEIN).

7.2.7.Test for glycosides

I..Keller- killani test.

It form redish color It indicate the absence of glycosides

7.2.8.Test for saponins.

I.Foam test

A 1 cm layer of foam indicates the presence of Saponins.

7.2.9 Test for terpinoid

observed for presence of reddish brown color interface to show positive results for the presence of terpenoid.

Table.NO:7:Qualitative phyto chemical screening of *Ixora coccinea* leaf extract (ICLE)

PLANT CONSTITUENT	INFERENCE
	Ethanol Extract
Carbohydrate	-
Alkaloids	-
Flavonoids	+
Proteins and amino acids	+
Glycosides	-
fixed oil	-
Terpenoids	+
Volatile oil	-
Tannins	-

“+” Presence, “-” Absence.

7.2. *In vitro* antioxidant activities

7.2.1. Effect of *ixora coccinea* leaf extract (ICLE) on superoxide radical scavenging activity

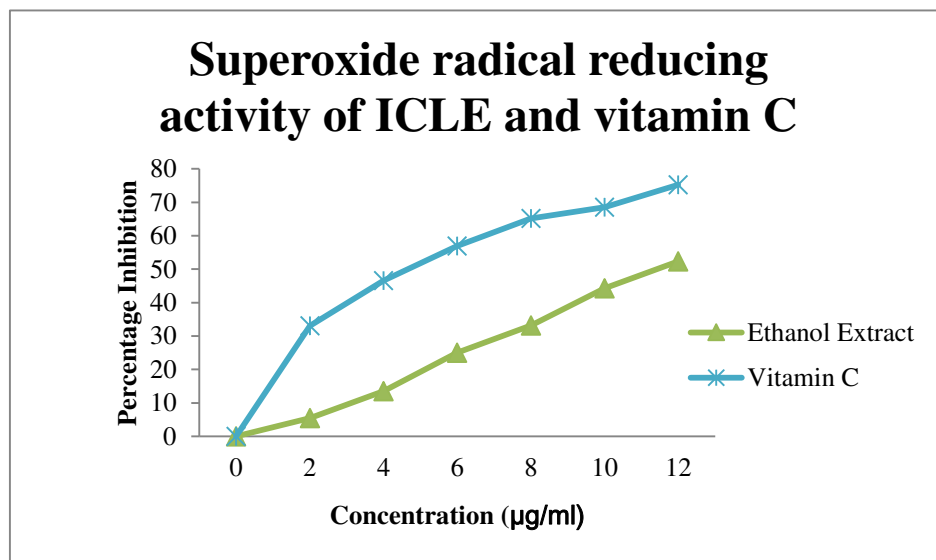
Superoxide generated in the photo reduction of vitamin C was effectively inhibited by the addition of varying concentrations (0-12 $\mu\text{L/ml}$) of extract table 8. The concentration of the ICLE needed to scavenge 50% superoxide anion (IC_{50}) was found to be 11 $\mu\text{g/ml}$ (figure 13) Vitamin C which was used as a positive control had an IC_{50} value of 4.5 $\mu\text{g/ml}$.

Table.No:8:Effect of ICLE on superoxide *in vitro* radical scavenging activity

Concentration ($\mu\text{g/ml}$)	Absorbance		Percentage inhibition	
	Ethanol Extract	Vitamin C	Ethanol Extract	Vitamin C
0	0.78 \pm 0.11	0.78 \pm 1.33	0	0
2	0.03 \pm 0.1	0.24 \pm 0.09	5.5 \pm 1.2	33.11 \pm 1.7
4	0.09 \pm 0.2	0.37 \pm 0.12	13.5 \pm 2.1	46.56 \pm 0.9 8
6	0.20 \pm 0.30	0.42 \pm 0.32	25 \pm 3.6	56.98 \pm 1.8 7
8	0.27 \pm 1.1	0.52 \pm 0.14	33.19 \pm 4.2	65.18 \pm 2.9
10	0.36 \pm 1.2	0.62 \pm 0.15	44.32 \pm 4.8	68.54 \pm 1.3 5
12	0.41 \pm 0.6	0.69 \pm 0.72	52.34 \pm 3.8	75.2 \pm 1.89

Results are mean \pm SD of three individual experiments

Figure. No:10: Superoxide radical reducing activity of ICLE and vitaminC



7.2.2.Effect of *ixora coccinea* leaf extract (ICLE) on DPPH radical reducing activity

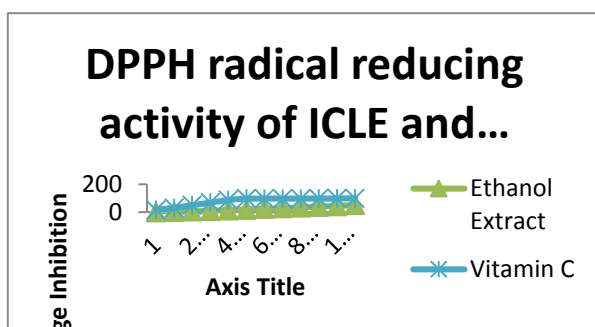
The DPPH radical was effectively scavenged by seed oil extract and Fraction-IV. A dose dependent reduction of was observed within the range of concentrations (0-100 $\mu\text{g/ml}$) of reaction system (Fig.14). The IC_{50} value of ICLE was found to be 110 $\mu\text{g/ml}$ (Table.9). Vitamin C which was used as the positive control exhibited an IC_{50} value of 21.6 $\mu\text{g/ml}$.

Table.No:9:Study of *in vitro* DPPH Radical Scavenging Activity of ICLE

Concentration ($\mu\text{L/ml}$)	Absorbance		Percentage Inhibition	
	Ethanol extract	Vitamin C	Ethanol Extract	Vitamin C
1	0.0691 \pm 0.12	0.591 \pm 0.23	0.21 \pm 1.2	15.8 \pm 1.23
10	0.686 \pm 0.14	0.231 \pm 0.68	2.33 \pm 0.22	29.9 \pm 1.45
20	0.665 \pm 2.5	0.341 \pm 1.5	5.1 \pm 1.22	49 \pm 1.63
30	0.640 \pm 2.3	0.231 \pm 1.23	8.9 \pm 1.5	67.8 \pm 3.76
40	0.59 \pm 1.56	0.22 \pm 1.85	13.5 \pm 6.5	87.3 \pm 4.56
50	0.57 \pm 2.3	0.132 \pm 1.44	18.6 \pm 4.22	97.2 \pm 7.6
60	0.53 \pm .05	0.062 \pm 1.56	24.5 \pm .3.89	96.1 \pm 5.67
70	0.501 \pm 0.51	0.059 \pm 1.23	28.9 \pm 2.3	96.2 \pm 7.89
80	0.472 \pm 2.5	0.052 \pm 2.56	33.4 \pm 0.45	96.2 \pm 1.23
90	0.0.437 \pm 1.02	0.04 \pm 0.06	38.9 \pm 1.23	97.5 \pm 1.96
100	0.407 \pm 3.8	0.03 \pm 0.03	42.1 \pm 3.4	97.6 \pm 3.45
120	0.331 \pm 5.6	0.02 \pm 0.01	53.1 \pm 1.56	98.3.6.56

Results are mean \pm SD of three individual experiments.

Figure. No:11: DPPH radical reducing activity of ICLE and vitaminC.



7.3 Pharmacological study

7.3.1.Acute toxicity studies

Acute toxicity studies on the sd rats show no mortality at adose of 2000mg/kg of ICLE during a time period of 14 days.during the study , no noticeable were seen in the rats . this help to predict that it does not contain any type of toxicity and it is full safe . so 200mg/kg between (1/10th dose) of ethanol extract were selected of that dose for the further study

7.4. Hepatoprotective activity

7.4.1. Effect of ICLE on SGPT concentration in paracetamol induced model

The results of biochemical parameters revealed to the alteration of enzyme levels in paracetamol treated group indicating that paracetamol induces damage to the liver. Table 10 shows that CCl₄ causes significant increase in SGPT level from control 93.22 ±1.22 IU/L to 157.22±0.16 IU/L after paracetamol intoxication. Administration of ICLE 200mg/kg and 400 mg/kg in paracetamol intoxicated rats caused reduction in SGOT level to 127.22±0.21 and 113.11±1.85IU/L respectively

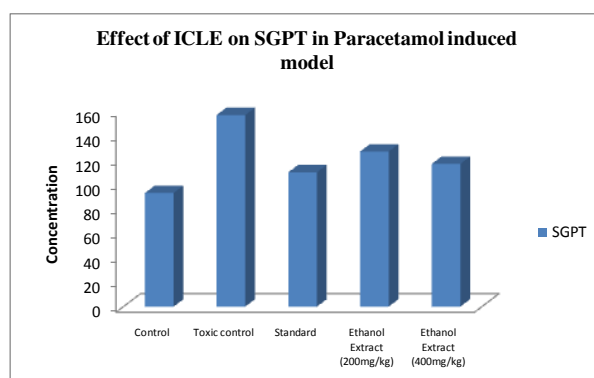
Table.No:10:Effect of ICLE on SGPT concentration in paracetamol

Treatment	Dose mg/kg	SGPT (IU/L)
Control	-	93.22±1.22
Toxic control	2 gm/kg	157.22±0.16
Standard	100mg/kg	110.22±0.19
Ethanol Extract	200 mg/kg	127.22±0.21
Ethanol Extract	400 mg/kg	113.11±1.85

induced model

.Value expressed as Mean ±SEM; Number of animals in each group
=6.*P<0.0001

Figure .NO:12:Effect of ICLE on SGPT concentration in paracetamol induced model



7.4.2.Effect of ICLE on SGOT concentration in paracetamol induced model

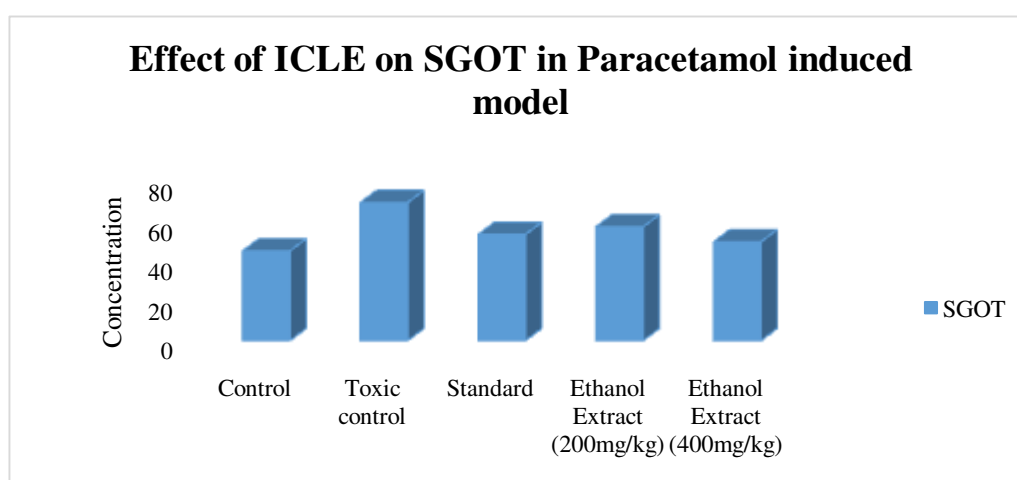
Table 11 shows that paracetamol affect significant increase in SGPT concentration from control 45.46 ± 0.24 IU/L to 70.05 ± 0.2 IU/L after paracetamol intoxication. Administration of ICLE 200 mg/kg and 400 mg/kg in paracetamol intoxicated rats caused reduction in SGOT level up to 56.21 ± 0.2 IU/L and 50.12 ± 2.2 IU/L respectively ($P < 0.001$). The extract in Standard dose of silymarine (100mg/kg) animals does not cause any significant changes in SGOT level

Table.No:11:Effect of ICLE on SGOT concentration in paracetamol induced model

Treatment	Dose mg/kg	SGOT (IU/L)
Control	Vehicle	45.46±0.24
Toxic control	2 gm/kg	70.05±0.2
Silymarine	100mg/kg	53.8±0.21
Ethanol Extract	200mg/kg	56.21±0.2
Ethanol Extract	400mg/kg	50.12±2.2

Values expressed as Mean ±SEM; Number of animals in each group =6. *P<0.0001

Figure .NO:13: Effect of ICLE on SGOT concentration in paracetamol induced model



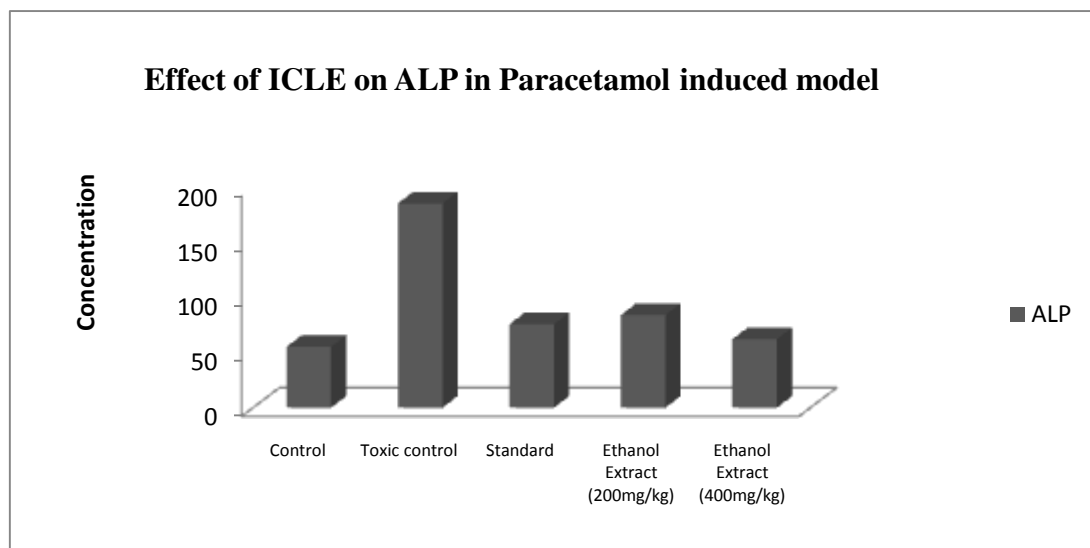
7.4.3. Effect of ICLE on ALP concentration in paracetamol induced model

ALP level in the control group increased from 55.02 ± 0.59 IU/L to 185.45 ± 0.22 IU/L in paracetamol intoxicated rat as shown in Table 12. Administration of ICLE 200mg/kg and 400 mg/kg in paracetamol intoxicated rats lead to lowering of the ALP level 84.12 ± 0.16 and 62.13 ± 1.27 respectively ($P < 0.001$). The extract in control animals showed no significant alteration in ALP level.

Table.No:12:Effect of ICLE on ALP concentration in paracetamol induced model

Treatment	Dose mg/kg	ALP (IU/L)
Control	Vehicle	55.02 ± 0.59
Toxic control	2 gm/kg	185.45 ± 0.22
Silymarine	100mg/kg	75.7 ± 0.21
Ethanol Extract	200mg/kg	84.12 ± 0.16
Ethanol Extract	400mg/kg	62.13 ± 1.27

Figure .No:14:Effect of ICLE on ALP concentration in paracetamol induced model



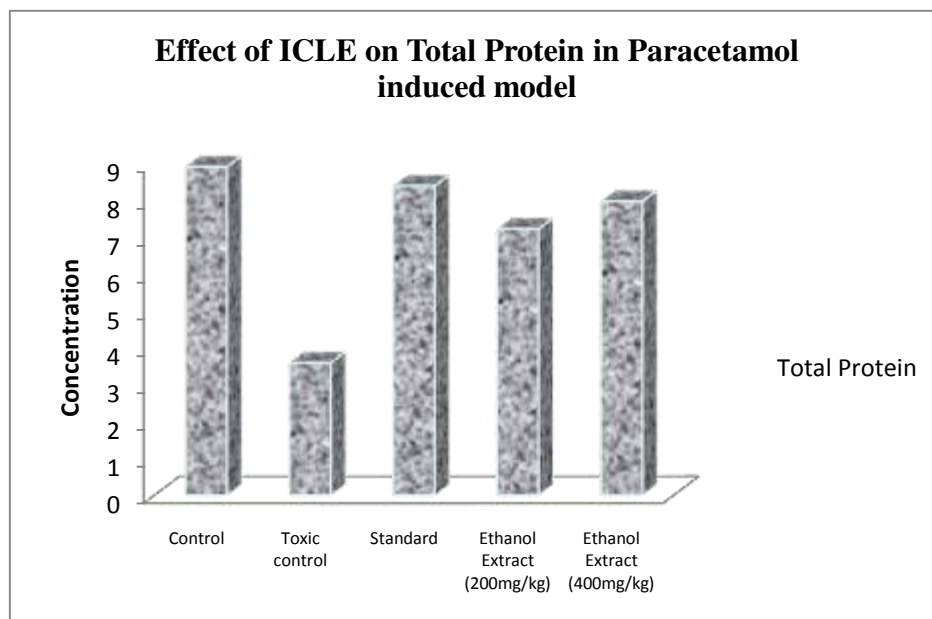
7.4.4.Effect of ICLE on Total Proteins in paracetamol induced model

Total protein level in the control group reduced from 8.93 ± 0.07 g/dl to 3.61 ± 0.08 g/dl in paracetamol intoxicated rat as shown in Table 13. Administration of ICLE 200 mg/kg and 400 mg/kg in paracetamol intoxicated rats led to increasing the concentration 7.24 ± 0.07 and 7.99 ± 0.03 respectively ($P < 0.001$).

Table.No:13:Effect of ICLE on Total Proteins in paracetamol induced model

Treatment	Dose mg/kg	Total Protein (g/dl)
Control	Vehicle	8.93 ± 0.07
Toxic control	2 gm/kg	$3.61 \pm 0.08\#$
Silymarine	100mg/kg	8.40 ± 0.06
Ethanol Extract	200mg/kg	7.24 ± 0.07
Ethanol Extract	400mg/kg	7.99 ± 0.03

Figure .No:15: Effect of ICLE on Total Protein in paracetamol induced model



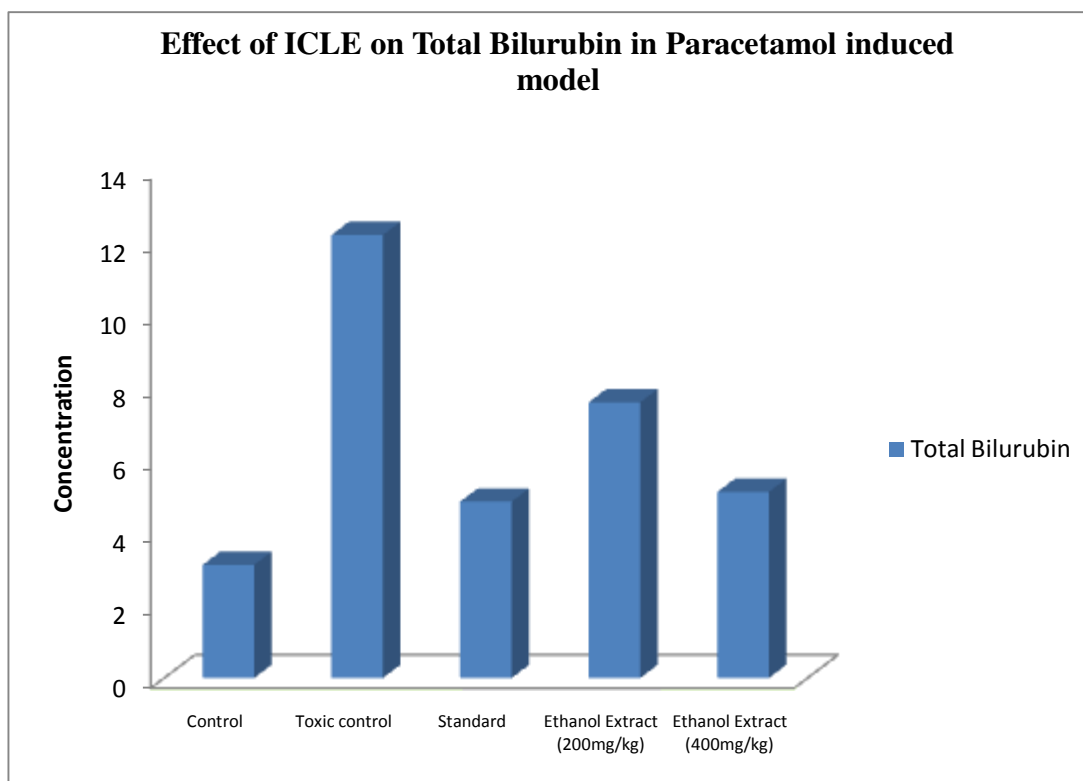
7.4.5. Effect of ICLE on Total Bilurubin in paracetamol induced model

Destruction of hemoglobin yields bilirubin which is conjugated in the liver to diglucoroxide and excreted in the bile. Bilirubin accumulates in plasma when liver insufficiency exists or biliary obstruction is present or rate of hemolysis increases. The total bilirubin level increased from 3.12 ± 0.02 mg/dL in the control group to 12.21 ± 0.04 mg/dL after paracetamol intoxication as shown in Table 14 Administration of ICLE 200 mg/kg and 400 mg/kg in paracetamol intoxicated rats reduced the total bilirubin to 7.60 ± 0.09 mg/dL and 5.13 ± 0.56 mg/dL ($P < 0.001$). However, the extracts in control rats showed no such significant alteration in the serum bilirubin level.

Table.No:14:Effect of ICLE on Total Bilurubin in paracetamol induced model

Treatment	Dose	Total
Control	Vehicle	3.12 ± 0.02
Toxic control	2 gm/kg	$12.21 \pm 0.04\#$
Standard	100mg/kg	4.86 ± 0.21
Ethanol Extract	200mg/kg	7.60 ± 0.09
Ethanol Extract	400mg/kg	5.13 ± 0.56

Figure .No:16: Effect of ICLE on Total Bilurubin in paracetamol induced model



7.4.6. Effect of ICLE on Body Weight for paracetamol induced model

Table 15 represents the body weight of different groups of rat treatment. The body weight of Paracetamol induced group II rats has been reduced comparing with group I. The body weight of Paracetamol induced group II rats has been reduced comparing with group I. After 28 days administration of silymarin and ixora coccinea on group III and group VI rats has gained the body weight almost to near normal level. The gain in body weight of group IV is compared with group II and it may be due to the regeneration of liver cells activity to near normal after the herbal treatment.

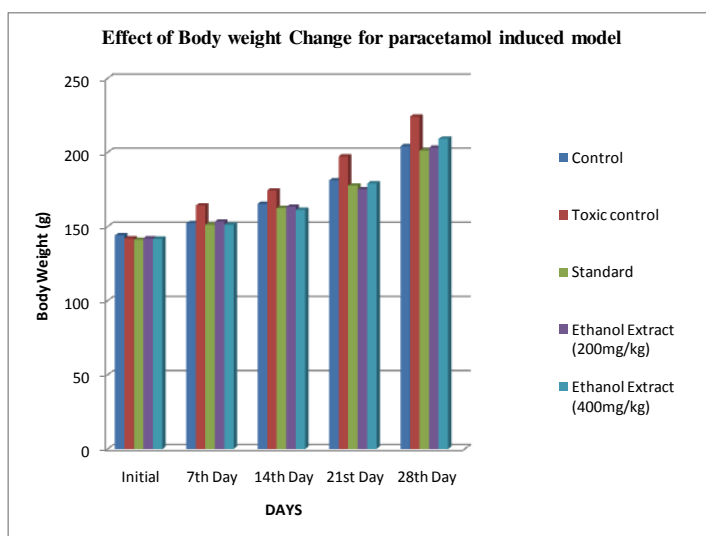
Table.No:15:Effect of ICLE on Body weight for paracetamol induced model

TREATMENT	DAY					% Change In Body Weight
	0	7	14	21	28	
Normal control	144.13±6.3 4	152.21±3.7	165.21±3.8 5	181.11±5.35	204.23±3.8	27.52%
Toxic control	142.19±6.8 5	164.39±5.2 0	174.25±4.2 1	197.3±2.3 3	224.13±6.2	35.87%
Silymarine (100mg/kg)	141.54±7.3 5	151.20±3.8	162.50±6.3 3	177.5±3.9 5	201.22±4.5	42.36%
Ethanol extract (200mg/kg)	142.32±4.5 5	153.20±3.3 3	163.22±2.4 4	175.21±3.75	203.44±1.37	44.37
Ethanol extract (400mg/kg)	141.55±6.0 3	151.11±1.4	161.33±2.9 1	179.14±2.2	209.14±2.5	47.78%

Values expressed as Mean ±SEM; Number of animals in each group =6

*P<0.0001

Figure .No:17: - Body weight change for Paracetamol induced model



7.5.HISTOPATHOLOGY

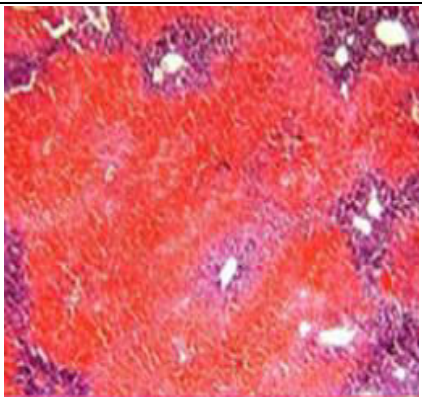
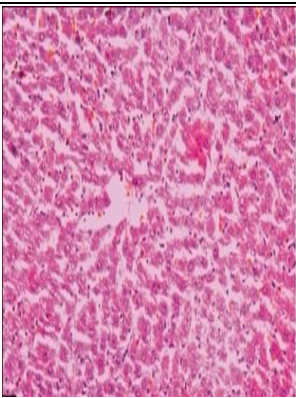
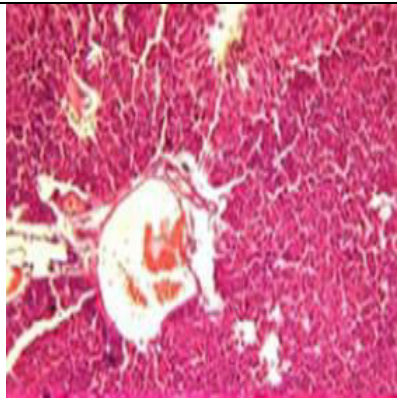
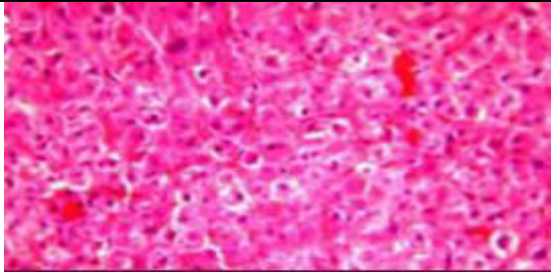
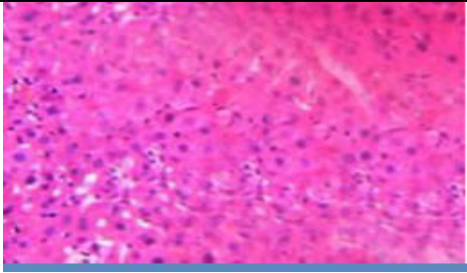
Histology is the study of the microscopic anatomy of cells and tissues of plants and animals. Histology is an essential tool of biology and medicine. Histopathology, the microscopic study of diseased tissue, is an important tool in anatomical pathology, since accurate diagnosis of liver diseases usually requires histopathological examination of samples.

The hepatoprotective effect of ICLE was confirmed by histological examination. The liver sections of normal control animals showed hepatic cells with well-preserved cytoplasm, prominent nucleus and central vein (Fig. 9 Group 1). The normal architecture of liver was completely lost in rats treated with Paracetamol (Fig. 9 – Group 2) with the appearance of vacuolated hepatocytes and degenerated nuclei. Vacuolization, fatty metamorphosis in the adjacent hepatocytes, cell infiltration of lymphocytes and Kupffer cells and necrosis of hepatocytes were severe in the centrilobular region and these changes were also observed in areas other than the centrilobular regions. The livers of rats treated with ICLE 100mg/kg, 200 mg/kg, 400mg/kg (Fig. 9 group IV-VI), and silymarin 100 mg/kg (Fig. 9 group III) showed a significant attenuation from paracetamol induced liver damage as evident from normal hepatocytes with well-defined nuclei.

Histological features

- Normal control shows normal cellular architecture with distinct hepatic cells, sinusoidal space and a central vein.
- Toxic control exhibited severe hepatocyte degeneration, fatty changes and necrosis.
- Silymarin shows mild hepatocyte degeneration.
- b ethanol extract shows normal architecture with mild hepatocyte degeneration through dose dependent

Figure .No:18: Histopathology study of ICLE

		
Group I Toxic Control	Group II Silymarine L	Group III Control
		
Group IV Ethanol Extract (200mg/kg)	Group V Ethanol Extract (400mg /kg)	

CHAPTER VIII

8.DISSCSSION

The plant *ixora coccinea* is widely distributed in suth asia . The s flower part of plant have been studied its antibacterial activity but the hepatoprotective effect have been never studied. Hence the objective of the study is determining this effect from the dried leaf extract of *ixora coccinea*

The preliminary phytochemical screening of whole plant extracts indicate in presence of flavanoid, terpenoids , protien and amino acid . may accounts antioxidant and hepatoprotective activity.

The antioxidant screening shows that that it showed reducing power to DPPH radicals. But the efficiency showed that far below from Vitamin C. The concentration of the ICLE needed to scavenge 50% superoxide anion (IC₅₀) equal to that of standard hence the leaf juice have the significant antioxidant activity

Liver is one of the important organs of body hence damage to liver leads to severe pathological problems or death. Liver diseases are mainly caused by toxic chemicals, excessive intake of alcohol, infections and autoimmune disorders. Liver injury caused by hepatotoxins such as carbon tetrachloride, ethanol and acetaminophen is characterized by varying degrees of hepatocyte degeneration and cell death by either apoptosis or by necrosis. SGOT, SGPT, ALP, Total Protein and Total Bilirubin levels are largely used as most common biochemical markers to evaluate liver injury.

Paracetamol (Acetaminophen), a widely used analgesic and antipyretic drug that produces acute liver damage in high doses. Paracetamol induced hepatotoxicity is thought to be caused by N-acetyl-p-benzoquinoneimine (NAPQI), a cytochrome P-450 mediated intermediate metabolite. NAPQI can react with sulphydryl groups such as glutathione and protein thiols. The covalent binding of NAPQI to cell proteins is considered the initial step in a chain eventually leading to cell necrosis. In paracetamol (PCM) treated acute hepatic injury a significant difference in biochemical markers was observed between normal and PCM control groups. Results of present study shows that

the levels of SGOT, SGPT, ALP and Total Bilirubin were significantly increased in paracetamol treated groups when compared with normal control group.

While total protein is decreased in paracetamol treated groups due to damage produced in endoplasmic reticulum. Comparative analysis on the effect of SGOT, SGPT, ALP and Total Bilirubin revealed that extracts shows marked decrease in these enzymes when compared with toxic control group.

While silymarine, and extract of ixora coccinea. Significantly restored total protein activities to normal level. Moreover, histopathological analysis showed that normal liver architecture was disturbed by paracetamol treated rats oral feeding with extracts shows normal architecture with mild hepatocyte degeneration compared with normal control group.

The preliminary phytochemical screening of whole plant extracts indicate in presence of flavanoid, terpenoids , protien and amino acid .

The antioxidant studies particularly showed that ICLE have slight antioxidant potential but that not inferior than standard vitamin C .

Hepatoprotective study results shows that the levels of SGOT, SGPT, ALP and Total Bilirubin were significantly improvement may accounts hepatoprotective activity

All these observation imply that the ICLE could be regarded as a favorable antioxidant and hepatoprotective agents.

As the results indicated that the extract possess significant hepatoprotective activity, after carrying out a thorough study of clinical trials, the plant can be considered as a low cost, potent, herbal medicine for liver disorders.

CHAPTER IX

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